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EFFECTS OF A VERY LOW DOSE RATE OF CHRONIC IONIZING RADIATION ON THE DIVISION POTENTIAL OF HUMAN EMBRYONIC LUNG FIBROBLASTS IN VITRO

F. CROUTE, S. VIDAL, J.P. SOLEILHAVOUP, C. VINCENT, G. SERRE, and H. PLANEL

Laboratoire de Biologie Médicale, Faculté de Médecine Purpan, 37, allées Jules Guesde, 31 000 Toulouse, France

Abstract—Among the various parameters that are supposed to play a role in aging at the cellular level, the "free radical theory" involves biochemical modifications that can be induced by radiation. Human embryonic lung fibroblasts were serially subcultivated at low density under chronic low dose rate irradiation (40 mrad/day) and in a normal environment. Irradiation increases cell attachment and the population doubling/day throughout their entire in vitro lifespan. Consequently, the doubling potential reached by irradiated cells was higher than that of control cultures. Finally, the total number of cells produced under chronic irradiation was 8–14 times higher than in a normal environment. These results are discussed with respect to the increased enzymatic activities (superoxidismutase, catalase, glutathion-reductase, G6PD) found in some irradiated organisms.

Key Words: cytogerontology, ionizing radiation, cell aging, radiation of cells

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HUMAN DIPLOID fibroblasts have a finite lifespan which can be related to the number of population doublings that have occurred in culture in vitro (Hayflick and Moorhead, 1961; Hayflick, 1965; Dell Orco *et al.*, 1973). This accounts for the use of diploid human cell cultures in exploring the aging mechanism at the cellular level.

Experimental changes in the lifespan of cultured human fibroblasts seem to be a promising approach to an understanding of the aging process. Thus, it has been observed that ionizing radiations have a life-shortening effect in many mammals (Walburg, 1975; Maisin *et al.* 1978; Sasaki and Kasuga, 1981) and in cultured cells, Macieira-Coelho *et al.*, 1977; Azzarone *et al.*, 1979; Ban *et al.*, 1981).

Similarities between the decline of growth in cell populations in vitro and that induced by radiation would tend to support the idea that ionizing radiation is able to accelerate natural aging at the cellular level. This assumption is based on the possibility that random damage, induced by free radical reactions generated continuously throughout cells from enzymatic and non-enzymatic reactions, or upon exposure to ionizing radiation, contributes in a similar way to the senescence and death of cells (Harman, 1981). In order to investigate the influence of background irradiation levels on lifespan, we have conducted

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experiments in which radiation was administered at a very low dose rate (40 mrad/day) during the entire in vitro lifespan of human embryonic lung fibroblasts.

MATERIALS AND METHODS

Cell culture

Human fibroblasts derived from embryonic lung as described by Hayflick and Moorhead (1961) and kindly supplied by Dr. Macieira-Coelho (Institut de Cancérologie et d'Immunogénétique, Villejuif, France) were used in the present study. Cells were grown in T-25 Falcon flasks, tightly closed and containing 8 ml of culture medium. The cultures were incubated at 37 °C in Eagle's M.E.M. supplemented with 10% fetal bovine serum (Biopro) and 20 μg/ml gentamycin (Sigma). The cells were removed from the plastic surface with 0.025% trypsin (Choay) in Ca^{++} and Mg^{++} free phosphate buffer at 37 °C for 2 min for counting and subcultivation. Cell counts were made with a Coulter Counter, model ZBI (Coulter Electronics Inc.).

Protocol for subcultivations

In each experiment, eight cultures were studied comparatively in a normal environment or under conditions of chronic irradiation. Four experiments were initiated using cells in phase II [at 21 or 25 population doublings (PD)] or in phase III (at 55 or 60 PD).

For subcultivation, cells were harvested with trypsin solution and suspended in growth medium in order to obtain a cell concentration of about 5×10^4 cells/ml. Cultures were initiated by inoculating 16 flasks with 8 ml of this cell suspension (eight controls, eight irradiated). Thus, the initial fixed number of cells per flask was about 4×10^5, i.e., 16×10^3 cells/cm^2. Twenty hours later, six of the flasks were used to estimate cell attachment (three controls, three irradiated).

With the remaining ten flasks, successive subcultures were made in an identical manner and performed when cells were actively growing, thus maintaining cultures in log phase growth throughout their entire in vitro lifespan (Kaji and Matsuo, 1980). The time between subcultures was determined though observation with an inverted microscope. Subcultivations were performed every 3 to 4 days during phase II, and at increasingly longer intervals during phase III.

At the time determined for each subculture, cells in the remaining flasks (five controls, five irradiated) were treated with trypsin and suspended in medium for counting (3 samples/flask and 4 counts/sample). The control or irradiated cells in the five flasks were pooled and a volume containing the characteristic inoculum (3.2×10^6 cells) was used to inoculate growth medium in order to perform the next subcultures (eight control, eight irradiated). The cells were counted again later in order to find the actual number of cells at the start of the culture.

Irradiation

The radioactive source was thorium nitrate crystals set in a thin and uniform layer under the cell culture flasks. Dosimetry was carried out by thermoluminescent detectors (LiF and Al_2O_3) (Commissariat à l'Energie Atomique, Fontenay aux Roses, France). The absorbed doses were about 40 mrad/day corresponding to 15 rad/year. As 200 mrad/year is the average natural irradiation dose absorbed by control cultures, the dose rate used was 75 times higher.

Estimation of cell attachment

Cell attachment was determined 20 hours after inoculation. For six flasks (three control, three irradiated), the medium was decanted and the remaining attached cells were removed by trypsin and counted (3 samples/flask and 4 counts/sample). The percent of cell attachment was calculated by dividing the number of attached cells by the initial cell number.

Estimation of population doubling (PD)

The number of cell population doublings was calculated at each transfer, taking into account the number of harvested cells. It was estimated either from the cell number inoculated and/or from the number of attached cells 20 hours after inoculation

$$PD = \frac{\log n \text{ harvested cells/inoculated cells or attached cells)}}{\log n\, 2}.$$

When the number of cells in a subcultivation fell below that of the inoculation, the cells were considered to

have stopped dividing and the cumulative number of doublings (doubling potential) was calculated. This value was taken as an index of the lifespan for the strain in culture.

Estimation of the generation time

The generation time was estimated from the number of population doublings and from the time elapsed between each subculture.

Assays

Protein content was determined according to Lowry's method (1951) using crystalline bovine albumin as a standard. Catalase activity was assayed according to the method of Beers and Sizer (1952), measuring the decrease in the absorbance at 240 nm over 5 min at 25 °C after addition of substrate (H_2O_2 final conc. 20 mM). The specific catalase activity is expressed in international units, i.e., in micromoles of hydrogen peroxide destroyed per minute per mg of protein.

The glucose-6-phosphate dehydrogenase (G6PD) activity was assayed by following the reduction of NADP at 340 nm according to the method of Lohr and Waller (1974). Units were expressed in *n*moles of NADP reduced/min/mg protein.

The glutathion reductase activity was assayed according to the method of Colman and Black (1965) measuring the rate of oxidation of NADPH followed at 340 nm. Units were expressed in nmoles of NADPH consumed/min/mg protein.

Statistical analysis

All the tests used were non-parametric tests. Comparisons of means were performed on paired samples by Wilcoxon's test and/or sign test and on unpaired samples by Mann's and Witney's test. The correlation was investigated using Spearman's rank correlation coefficient.

RESULTS

1. The cell attachment

Cell loss at each subcultivation is thought to be an important factor influencing the apparent maximum population doubling level reached by a cell population. We evaluated cell loss from counts of the attached cells 20 hours after each subcultivation. Figure 1 shows the results of one experiment using cells at 21 population doublings when the irradiation was started. It indicates that the ability of the cells to attach gradually decreases with age in control cultures as well as in irradiated cultures. The coefficient of Spearman's test is -0.825 in control cultures and -0.720 in irradiated cultures. In both cases the associated risk is <0.01.

On the other hand, we observed that ionizing radiation seems to increase cell adhesiveness throughout the lifespan of the cultures. Although the difference found between control and irradiated cells is slight, and not always significant for each point (test of Mann and Witney), a comprehensive study using Wilcoxon's test shows that the difference was significant (<0.02) in the two experiments performed with cells at 21 or 25 PD. However, in the two experiments where the irradiation was started on cells in phase III (56 or 61 PD), no significant difference between control and irradiated cells has been observed.

The 0.20% increase of irradiated cell numbers found between the 18th and 38th subcultures (Figure 1) can be explained by the fact that subcultivations were performed in actively growing cultures in which cells were in various phases of their cell cycle. Thus, a fraction of the cells may divide within the 20 hours following the subcultivation. Consequently, the increased cell number found under irradiation could be due to growth stimulation rather than to an enhanced adhesiveness. These explanations agree with

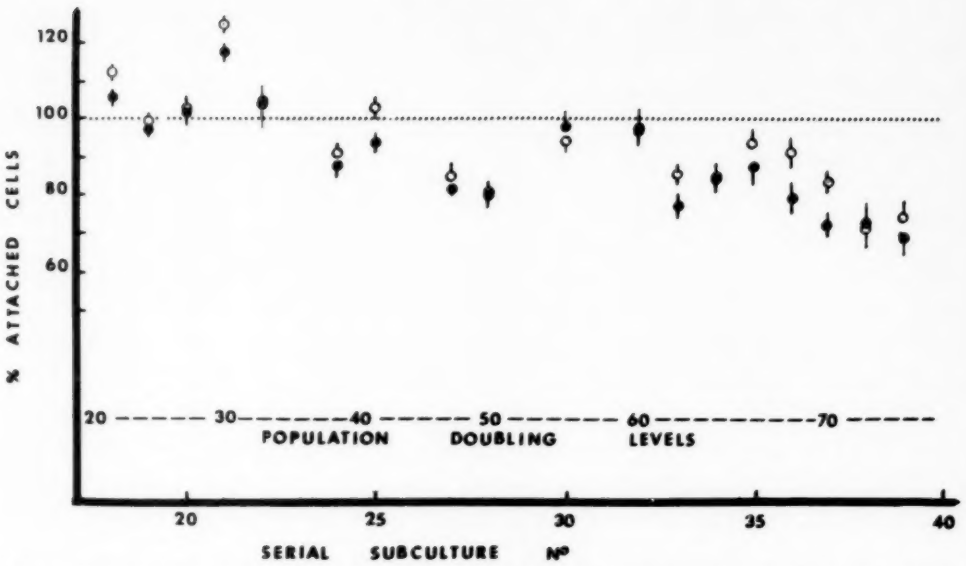

FIG. 1. Percentage of attached cells 20 hours after inoculation at various population doubling levels. Control cultures (●) irradiated cultures (○). Vertical bars show standard deviations.

Werry's and Stoffelsen's observations (1979) showing that, up to doses of about 4 Gy of X-rays, the plating efficiency of irradiated *Haplopappus gracilis* cells is higher than that of control cells. Moreover, we checked that the preliminary irradiations of the plastic culture flasks and of the growth medium do not cause any significant changes in the cell attachment.

2. *Population doubling/day*

Since the time elapsed between two successives subcultures varies and especially increases progressively with age, we calculated the number of population doublings/day (Figure 2) in order to compare them. In control and irradiated cultures, population doublings/day were roughly constant (approximately 0.6–0.8 PD/day) during phase II and decreased quickly in phase III, i.e., during the last 4–5 subcultures.

When young cells were irradiated, after 21 PD (Figure 2) or after 25 PD (data not shown, but quite comparable), the number of doublings/day was higher than in control cultures. Wilcoxon's test shows that the difference was significant (< 0.01). When irradiation was started on aged cells (55 or 61 PD) (Table 1), we noted a very slight increase of the number of doublings/day during the first subcultures followed by a decrease of doubling number in irradiated cells.

3. *The doubling time*

The doubling time was calculated from the time elapsed between each subculture and from the number of PD accomplished. In all the experiments, the doubling time evolves in a similar manner throughout the in vitro lifespan (Figure 3A). During phase II, the gener-

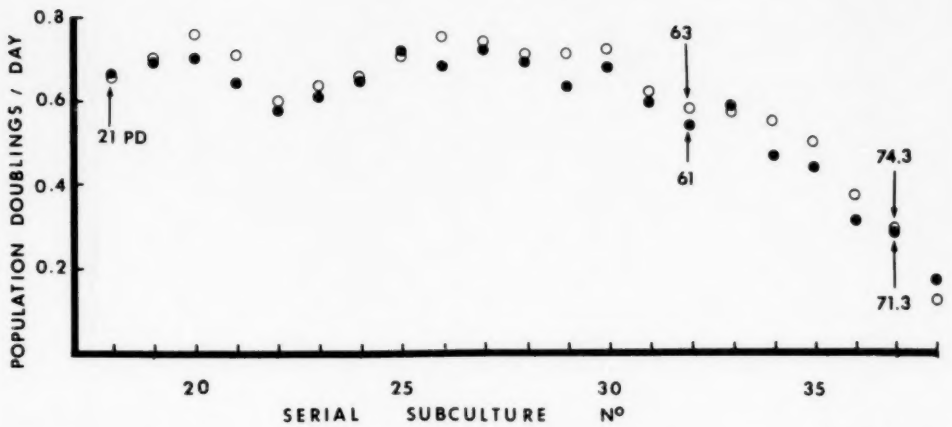

FIG. 2. Changes in the number of population doublings/day throughout the lifespan of young fibroblasts (21 PD) serially subcultivated in a normal environment (●) or under chronic irradiation (○). Error bars of ±2 S.D. are not shown because it does not exceed the size of the point.

ation time was approximately 35 hours. Then, as the in vitro age progressed to the senescent phase, the doubling time greatly increased.

Under chronic irradiation, we can observe a significant shortening of the generation time when irradiation started at 18 PD (Figure 3A) or 25 PD (Wilcoxon's test <0.01 for both experiments). On the contrary, in the experiments using cells at 56 or 62 PD, irradiation caused an increase of the generation time when cells entered the senescent phase (doubling time increasing).

4. The doubling potential

Figure 3B and Table 2 show the number of doublings achieved by cells in the four experiments. To compare the total number of population doublings accomplished by con-

TABLE 1. VARIATIONS IN THE NUMBER OF POPULATION DOUBLINGS/DAY OF AGED FIBROBLASTS (56 OR 61 PD AT THE START OF EXPERIMENTS) IN A NORMAL ENVIRONMENT OR UNDER CHRONIC IRRADIATION.

	Population Doubling/day of culture ± S.D.			
Subcultures N°	*Control*	*Irradiated*	*Control*	*Irradiated*
56	0.572 ± 0.007	0.603 ± 0.007		
57	0.563 ± 0.009	0.593 ± 0.003		
58	0.521 ± 0.010	0.524 ± 0.006		
59	0.462 ± 0.008	0.481 ± 0.010		
60	0.529 ± 0.008	0.513 ± 0.010		
61	0.366 ± 0.007	0.264 ± 0.010	0.481 ± 0.005	0.514 ± 0.005
62	0.248 ± 0.003	0.203 ± 0.005	0.352 ± 0.010	0.361 ± 0.006
63	0.167 ± 0.005	0.094 ± 0.004	0.288 ± 0.080	0.261 ± 0.009
64			0.137 ± 0.005	0.084 ± 0.005
65			0.051 ± 0.005	

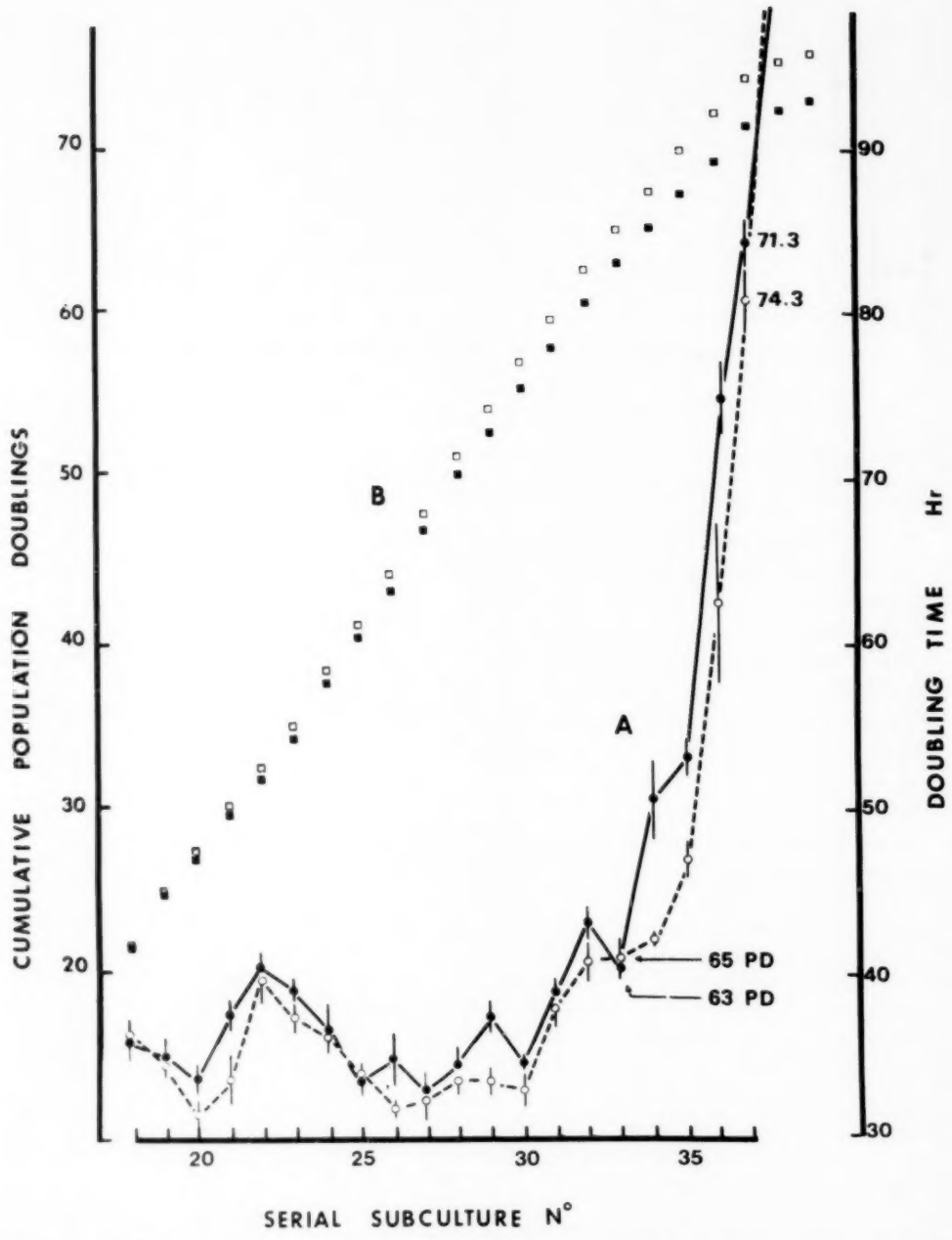

FIG. 3. A: Variations in the population doubling time of cells serially subcultivated. Control cultures (●), irradiated cultures (○). Vertical bars show standard deviations. B: Cumulative growth curves of cells serially subcultivated in a normal environment (■) or under chronic irradiation (□).

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TABLE 2. CHANGES OF THE DOUBLING POTENTIALS UNDER CHRONIC IRRADIATION OF CELLS OF DIFFERENT AGES WHEN THE IRRADIATION WAS STARTED. VALUES CORRESPOND TO THE CUMULATIVE NUMBER OF PDs CALCULATED FROM THE NUMBER OF INOCULATED (*) OR ATTACHED CELLS (**) AT EACH SUBCULTURES.

	Doubling potential		
PD	*Control*	*Irradiated*	*Sign test 2a*
21^{*}_{**}	69.90 72.20	73.70 75.60	<0.01 <0.01
25^{*}_{**}	70.27 72.17	72.21 74.80	<0.05 <0.05
56^{*}_{**}	68.52 69.68	67.81 68.43	N.S.
61^{*}_{**}	70.01 70.69	68.77 69.09	N.S.

trol and irradiated cultures in vitro, we used the sign test. For each subculture, the difference in the number of doublings between control and irradiated cells was noted +, −, or 0 using the Mann's and Witney's test. The sign test using these new data shows that the differences observed between the number of doublings of control and irradiated cultures become significant after 16 subcultures performed under chronic irradiation (experiment using cells at 21 or 25 PD).

In the experiments starting with cells at 56 or 61 PD, the small number of subcultures studied under irradiation is not sufficient to allow affirmation of a significant difference. Consequently, in the experiments using young cells (21 or 25 PD) irradiated cells grow more rapidly and their doubling potentials are greater than those of control cells. Under these conditions the final number of cells produced under chronic irradiation in the two experiments is approximately 8–14 times higher than in a normal environment.

On the other hand, we observed, in our culture conditions, that cells reached the end of their in vitro lifespan after 20 to 22 subcultures after the 21st or 25th PD, i.e., 3 months later. This result agrees with those of Kaji and Matsuo (1980) showing that cells, subcultivated at low density, have their calendar time in culture shortened compared with the lifespan of cultures subcultivated at a 1:4 split ratio without decreasing the cumulative number of PD achieved.

5. Enzyme assays

As enzyme activities were seen to vary greatly throughout the lifespan of the cells and no correlation with aging was apparent, we investigated the activities of certain enzymes between an inoculation and the end of that subculture. The result, given in Table 3, clearly shows how the activities are closely linked to the number of days in culture and to the growth phase of the cells (for cells at 25 and 41 PD).

Table 3 shows that catalase and G6PD activities varied greatly throughout the growth curve of cultures, first decreasing to the third day and then strongly increasing during the log growth phase. The fluctuations observed when assays were performed at the time of each subculture can be explained by the fact that, with our experimental protocol, the time elapsed between inoculation and the next passage has varied during the lifespan.

Nevertheless, if we compare the enzymatic activities of control and irradiated cultures throughout the growth curve we can note that the levels of catalase and G6PD activities were higher in the irradiated cultures.

Glutathion reductase activities increased during log phase growth to 1 to 13 I.U./mg protein and remained roughly constant during the plateau phase. A significant increase of activity levels in irradiated cultures appears only on the third and fourth day of culture.

DISCUSSION AND CONCLUSION

It is interesting to note from our observations that ionizing radiation shows opposite effects to those usually reported (Walburg, 1975; Maisin *et al.*, 1978; Azzarone *et al.*, 1979; Ban *et al*, 1981; Sasaki, 1981).

We have found that chronic irradiation at a very low dose rate involves both an increase of the doubling potential and a shortening of the doubling time. Consequently, the total amount of cells produced in irradiated cultures is larger than in controls. The results vary according to whether irradiation starts early or late during the lifespan (Table 2). These data agree with those of Macieira-Coelho *et al.* (1977) showing that when irradiation is applied to early PDL human embryonic lung fibroblasts, it can either have no effect or it can slightly extend the cell population lifespan. When irradiation is applied late, the number of doublings remains unchanged.

In the literature, a few authors have reported studies using chronic low-dose irradiation. De *et al.* (1983) showed that chronic whole body irradiation (10 rad/day) accelerated the aging process in mice. Similarly, Tixador *et al.* (1981) reported that exposure of paramecia to 2 mrad/day during the 90 days of their life led to a shortening of their lifespan.

Using acute (100 rads) or fractionated doses (5 times 100 rads) of low dose rate ionizing radiation (0.28 rad/min), Lima *et al.* (1972) showed that the growth decline started earlier in irradiated cultures of chick embryonic fibroblasts. In contrast, it has been reported that similar radiation doses accelerated the acquisition of an infinite growth potential in mouse fibroblasts (Macieira-Coelho *et al.*, 1976a) and increased the growth potential of human fibroblasts from donors with certain genetic diseases (Diatloff and Macieira-Coelho, 1979). Human lung fibroblasts showed an intermediate response since ionizing radiation neither established the culture as occurs with mouse cells, nor reduced the number of cells produced, as this occurs in chicken fibroblasts (Macieira-Coelho *et al.*, 1976b).

It is difficult to explain the different responses of these three species of fibroblasts. Macieira-Coelho (1976b) hypothesizes that in the case of mouse fibroblasts there are some cells in the original population capable of dividing indefinitely. If these cells are less radiosensitive, ionizing radiation could favor their overgrowth by killing or slowing down the growth rate of other cells in the population which have a limited doubling potential. In chicken fibroblast populations, cells with an infinite doubling potential cannot be found. Among human fibroblasts there could be clones with different growth potentials and ionizing radiation could favor clones with the longest division potential.

In our work, it is difficult to assume that the very low dose of ionizing radiation used

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TABLE 3. ASSAYS PERFORMED THROUGHOUT A GROWTH CURVE OF CONTROL AND IRRADIATED CULTURES CARRIED OUT WITH CELLS AT 25 PD OR 41 PD AT THE START OF THE EXPERIMENT. EACH VALUE IS THE AVERAGE OF TRIPLICATE DETERMINATIONS ± S.D.

Days of culture		*Catalase*		*G6PD*		*Glutathion Reductase*	
		Control	*Irradiated*	*Control*	*Irradiated*	*Control*	*Irradiated*
2	25 PD	8.51 ± 0.21	8.62 ± 0.34	95.70 ± 1.75	101.70 ± 1.52	1.52 ± 0.12	1.47 ± 0.15
	41 PD	7.77 ± 0.30	8.49 ± 0.42	100.58 ± 1.51	105.31 ± 1.13	1.10 ± 0.09	1.09 ± 0.10
3	25 PD	5.56 ± 0.33	6.33 ± 0.58	61.64 ± 1.82	72.51 ± 2.07	2.52 ± 0.18	3.20 ± 0.11
	41 PD	6.98 ± 0.44	8.07 ± 0.63	79.34 ± 1.61	82.66 ± 1.72	2.20 ± 0.13	2.47 ± 0.12
4	25 PD	10.23 ± 0.21	10.77 ± 0.24	105.25 ± 3.09	125.80 ± 1.52	8.44 ± 0.21	13.50 ± 0.25
	41 PD	11.16 ± 0.55	11.63 ± 0.30	112.36 ± 2.93	118.31 ± 2.50	9.38 ± 0.23	10.04 ± 0.20
5	25 PD	15.95 ± 0.31	19.31 ± 0.43	146.52 ± 4.06	177.76 ± 3.91	5.81 ± 0.15	6.16 ± 0.14
	41 PD	16.74 ± 0.30	16.98 ± 0.24	175.64 ± 3.15	211.65 ± 3.20	6.00 ± 0.12	5.18 ± 0.18
6	25 PD	13.90 ± 0.34	14.50 ± 0.15	170.22 ± 2.13	175.36 ± 2.03	8.76 ± 0.17	8.64 ± 0.21
	41 PD	13.89 ± 0.25	14.05 ± 0.46	186.51 ± 2.30	193.50 ± 2.42	7.46 ± 0.19	7.27 ± 0.21

could lead to the death of some cells since we found both an enhancement in the cell attachment and an increase in cell proliferation throughout the entire phase II of the in vitro lifespan in the irradiated fibroblasts.

The stimulating effect of low dose rate ionizing radiation on cell growth has been reported in bacteria (Eugster, 1964; Conter *et al.*, 1983), protozoa (Planel *et al.*, 1979; Croute *et al.*, 1982) and malignant human permanent lines (Croute *et al.*, 1971). Furthermore, it has been observed that such irradiation increases the growth potential of human fibroblasts from donors with certain genetic diseases (Diatloff and Macieira-Coelho, 1979). Moreover, Kiefer (1974) reported that energy metabolism always increased in yeast after low doses of irradiation.

Because of the very low dose used in our studies, we believe that the stimulating effect is a result of intracellular enzymatic scavenging of radioproduced H_2O_2 generated either by OH recombination or by chemical or enzymatic dismutation of O_2^- (Croute *et al.*, 1982). Indeed, Oberley *et al.*, (1980) hypothesize that the signal for cell division involves an increase in intracellular H_2O_2 which activates the membrane glucose carrier (Taylor and Halperin, 1979). H_2O_2 can also react with reduced glutathion (GSH) through glutathion peroxidase and the reversible oxidation of GSH by NADPH-dependent glutathion reductase produces $NADP^+$ regeneration which is a limiting factor in the oxidative phosphate pathway (Osmond and Aprees, 1969; Thomas, 1979). Consequently the $NADP^+$-dependent G6PD activity is enhanced thus giving rise to stimulation of the pentose phosphate shunt which contributes to the additional production of ribose-5-P as a precursor for RNA synthesis. This interpretation is in good agreement with results showing a stimulation in the hexose monophosphate shunt activity of irradiated lymphocytes (Roberts *et al.*, 1979) and an increase of RNA synthesis in irradiated plants (Niemann *et al.*, 1976).

Our investigations support these considerations insofar as we have found an increase of glutathion reductase and G6DP activities in irradiated bacteria (unpublished data). Similarly, in our laboratory, Conter (1985) showed that superoxidismutase and glutathion reductase are enhanced in irradiated algae. In fibroblasts, assays performed over a complete growth curve show that catalase, glutathion reductase and G6PD activities were increased under irradiation (Table 3).

These results also show that the enzyme activities are not identical, depending on the moment of observation during the growth curve. Because of the variability of the time elapsed between each subculture, no correlation can be observed between enzyme activities and the aging of cells.

On the basis of present data, it is not unreasonable to suggest that the enhancement of these enzyme activities depresses the adverse effects of free radicals radioproduced or generated from metabolic reactions in the cells, which lead to the increase observed in the doubling potential.

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USE OF MOTOR PERFORMANCE TESTS TO DIFFERENTIATE AGING EFFECTS FROM DISEASE EFFECTS

JOHN E. OTTENWELLER, WALTER N. TAPP, and BENJAMIN H. NATELSON*

Primate Neuro-behavioral Unit (127A), Veterans Administration Medical Center, East Orange, New Jersey 07019 and Department of Neurosciences, University of Medicine and Dentistry-New Jersey Medical School, Newark, New Jersey 07103

Abstract—The present study has demonstrated that behavioral testing can provide non-invasive methods for monitoring the time-courses of aging and disease processes. Performance on two of our tests changed linearly with increasing age in both cardiomyopathic and healthy hamsters, but these changes occurred at an earlier age in the CM hamsters than in healthy control hamsters. In addition, healthy hamsters showed age-related changes in performance on some tests, while sick CM hamsters did not show parallel changes. These results indicate that disease can modify the rate of change in some markers of aging, and thus they suggest that disease might be able to modify the aging process. However, such a preliminary hypothesis requires much further work. Performance on another behavioral test was shown to be stable throughout much of the lifespan of healthy hamsters, and yet disease in CM hamsters was clearly able to affect it. Thus it was possible to find some tests which discriminated between aging and disease effects, but only because the time-course of the disease we were studying was well defined. Most importantly, these results have suggested a way of thinking about the problem of disease in aging studies that may be more fruitful than others previously used. Examining the changes over time in both healthy subjects (putatively disease-free) and those with disease should allow one to determine which age-related changes are dependent on the presence of organic disease and to separate these from the changes which might inevitably occur solely from increasing age.

Key Words: aging and disease, motor performance, cardiomyopathy, aging in hamsters

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AS MAMMALS age, their susceptibility to disease increases; and when they finally die, organ pathology is usually found. Therefore, aging and disease often seem inextricably tied to one another. This interaction makes it difficult to decide whether differences in "biological age" are due to differences in the aging process *per se* or whether they are totally unrelated to "normal aging" but instead are a function of altered disease incidence or severity. A number of studies have sought to solve this dilemma by comparing the biological ages of people with either diagnosed or self-perceived health problems to those of healthy controls (Furukawa *et al.*, 1975; Webster and Logie, 1976). But Ingram (1983) points out the circularity in this experimental strategy: the increases in biological age in

*Requests for reprints should be addressed to Dr. Natelson at the Primate Neuro-behavioral Unit.
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the unwell sub-sample chosen may be due to the fact that it is "merely sicker than the normal sample." Costa and McCrae (1980) deal with the problem by suggesting that aging be defined to exclude disease and pathology, but this may lead to underestimating the possibility of covert disease in gerontological studies. Moreover, the occurence of disease (especially covert disease) sometime during the life of an animal can never be completely eliminated, and if it could, how relevant would this be to the human situation?

In thinking about this problem, we wondered whether some of the same tactics that have been used to study aging might also be used to study disease, and then based on similarities and/or differences between healthy and diseased populations, perhaps a start could be made at loosening the Gordian knot between aging and disease. To do this, we undertook to study two groups of hamsters over time: healthy hamsters and cardiomyopathic (CM) hamsters—those with an inherited generalized muscular disease which produces death due to progressive heart failure by 12–15 months of age. These animals have disease in both skeletal and cardiac muscle. While the former is mild but gradually progressive, the cardiac lesions develop only when the hamster is young (i.e., 2 to 4 months old). Thereafter, however, the heart goes through an orderly but inexorable process of hypertrophy and dilatation producing first a protracted period of compensated heart failure followed by uncompensated failure which rapidly ends in death.

Because of its relative simplicity and non-invasiveness, the tactic used by researchers studying aging that appealed most to us was motor performance testing. A number of groups have shown orderly changes in such tests for mice (Dean *et al.*, 1981; Ingram *et al.*, 1982; Ingram, 1983) and rats (Janicke *et al.*, 1983; Miquel and Blasco, 1978; Wallace *et al.*, 1980) over the course of their lifetimes. Our plan was to adapt existing behavioral tests of motor performance for use with hamsters as well as to develop other behavioral tests to assess these animals. By assessing a wide variety of tasks in our two populations of hamsters, our goal was to find some that tracked chronological age in both strains and others that were not related to age but did change when CM hamsters had overt evidence of their disease process. If we were able to develop such tests, they would be important for monitoring aging and disease processes in subsequent studies of healthy hamsters as they approached the end of their lives either "naturally" or due to some experimental intervention. Such tests would also allow us to compare the rates at which performance on them either increased or decreased with age and thus we could compare rates of aging in healthy and diseased hamsters to determine if disease interacts with the aging process. This paper reports that we were able to find performance tasks that tracked aging and/or disease. By defining the effect of disease on performance, we were also able to show definitively that some age-related changes were unrelated to general debilitation due to chronic disease.

MATERIALS AND METHODS

Young mature male cardiomyopathic (CM; BIO 14.6) and control (BIO F1B) hamsters were obtained at approximately 3 months of age from Bioresearch Inc., Boston, MA. These two inbred strains of hamsters were housed in our animal quarters for varying lengths of time before being used, i.e., the hamsters studied at 6 months of age were in our facility for 3 months, those studied at 12 months for 9 months, and the very old hamsters studied at 30 months of age were housed for 27 months. All behavioral studies were performed concurrently on hamsters which had arrived in the laboratory at different times. The hamsters were maintained throughout their lives on a 12h:12h photoperiod with the temperature maintained at 23 ± 2 °C and given free access to Purina Mouse Chow and to tap water. CMH hamsters were studied at 6 and 12 months of age, i.e., an age when the animals were compensating well for any damage to their hearts (6 months) and an age when the CM hamsters were overtly sick and in severe heart failure (12 months). Age-matched controls were studied at 6 and 12 months of age, and 30 month controls were studied because they were approaching the end of their lifespans

as were the 12 month CM hamsters. In our laboratory, the median lifespans for CM and healthy hamsters are 13 months and 27 months, respectively. We believe the 30 month old controls were in fact healthy because they lived for 4, 5, 8, and 12 months after the completion of this study.

Six hamsters comprised each group except for the 30 month group which consisted of four hamsters. All hamsters were tested for performance on the same set of behavioral tests for five consecutive days. The following week, two more days of testing were performed. In many tests, the performance during the first week showed consistent trends which stabilized by the end of the week. Therefore, the results of the two days of testing in the second week were analyzed.

Table 1 presents an outline of all the behavioral tests to which the hamsters were subjected. Although all tests are listed for completeness and as a guide for other researchers, all hamsters performed similarly on Test 5 and the last four tests, and these will not be discussed further except to note that some of these tests have been shown to change with aging in rats. The results for the last two days of testing were averaged and either logarithm or square root transformed to normalize the data. The transforms were analyzed by Analyses of Variance with individual group comparisons made by Dunn's tests (Kirk, 1969). Least squares linear regression was used to examine some changes in performance with age (Sokal and Rohlf, 1969). Finally, the frequency with which hamsters performed a particular way on some tests (e.g., the number which fell on the elevated platform test) were compared among groups using Chi-square tests (Sokal and Rohlf, 1969).

RESULTS

Figure 1 presents data from tests which were affected by age in both healthy and CM hamsters. An important thing to note is that the slopes of performance of these tests over time differ and that therefore an interaction between aging and disease appears to exist. The left panel (1A) presents the average number of escape attempts made by hamsters placed in an open bowl (Test 8). Healthy hamsters made progressively fewer escape attempts as they aged, and this linear decline was statistically significant ($r = 0.50$, $F = 4.79$ w 1,14 df, $p < 0.05$). Analysis of variance showed that 6 and 12 month healthy hamsters made a similar number of escape attempts ($p < 0.05$, Dunn's). Young CM hamsters attempted to escape as often as either 6 or 12 month healthy hamsters. However, 12 month CM hamsters made fewer escape attempts than either the younger CM hamsters or age-matched healthy hamsters ($p < 0.05$), and their number of escape attempts was similar to the older 30 month healthy hamsters.

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The right panel of Figure 1 (1B) presents the mean times required for the hamsters to descend from the top of an inclined screen (Test 2). In healthy hamsters, the time required for descent increased monotonically over the three ages studied, and this trend was statistically significant ($r = 0.82$, $F = 27.88$ w 1,14 df, $p < 0.01$). Again the 6 and 12 month healthy hamsters behaved similarly, whereas the 30 month healthy hamsters required significantly longer to descend ($p < 0.01$, Dunn's). Overall, CM hamsters took longer to descend than healthy controls ($F = 32.20$ w 1,20 df, $p < 0.001$), thus indicating that strain affected performance on this test. The 12 month CM hamsters took longer to descend the screen than 6 month CM hamsters ($p < 0.01$, Dunn's). The results of this test were qualitatively similar, albeit in the opposite direction, to those from the first test, and as was the case for the first test, suggested a possible interaction between aging and disease.

Figure 2 presents the results of those tests in which aging affected performance in healthy hamsters but not in CM hamsters. Therefore, disease may also interact with normal aging by preventing some changes in performance that normally occur with aging. In all four of these tests, 2 × 2 analyses of variance for 6 and 12 month hamsters revealed significant aging effects ($p < 0.05$) which were due to significant differences between the performances of 6 and 12 month healthy hamsters ($p < 0.05$, Dunn's). On the horizontal wire test (Test 1, Figure 2A), 30 month healthy hamsters continued the decline in the time

TABLE 1. BEHAVIORAL TEST PROCEDURES

1. Prehensile Reflex (Horizontal Wire Test)—The hamster was placed in a vertical position so that its front paws were grasping a wire strung 15 inches above the floor. The time that the hamster was able to remain suspended was recorded (in seconds). The time limit on this test, and all subsequent ones, was set at 3 minutes (180 seconds).
2. Descent of Inclined Screen—The hamster was placed at the top of a 45 degree inclined screen that was 30 inches long. The screen had one-quarter inch mesh. The time required (in seconds) for the animal to reach the bottom of the screen was recorded. A barrier was placed at the top of the screen in order to prevent the animal from climbing over the top. If the hamster did not succeed in the test, a default time of 180 seconds was recorded.
3. Middle of Inclined Screen—The hamster was placed in the middle of the inclined screen used in the previous test. The time required for the hamster to reach either the upper or lower bound of the screen was recorded. The final direction that the hamster took, i.e. either up or down, was also recorded (3b). If the hamster did not reach either of the specified boundaries within 180 seconds, the time recorded was 180 seconds and no direction was entered.
4. Latency to Descend Vertical Pole—The hamster was placed atop a three foot vertical pole which was wrapped in a one-quarter inch wire mesh. The time (in seconds) that was required for the hamster to descend the pole and reach the ground was recorded. If the hamster did not reach the ground, a default time of 180 seconds was established. Also whether the hamster climbed down the pole or dropped was also entered (4b).
5. Elevated Platform—The hamster was placed on the center of a one inch wide horizontal plank that was 30 inches long and suspended between two platforms. The time required for the hamster to walk to either of the platforms or to fall off the plank was recorded, as was whether the hamster fell or walked (5b). A default time of 180 seconds was also used in this experiment.
6. Time to Escape Container—The hamster was placed under a small plastic beaker in an 2′ × 2′ area enclosed by a wire that was four inches off the ground. When the animal moved the beaker to the wire, the beaker tipped over and the animal had escaped. The time required for the animal to escape the container was recorded (Test 6), and a default time of 180 seconds was used. During the time that the animal spent under the beaker, the number of rears (Test 6b, the hamster standing up on his hind legs) and the number of complete 360 degree turns (Test 6c) made by the hamster were noted and recorded.
7. Plexiglass Slide—The hamster was placed on a horizontal sheet of plexiglass. The angle of the plexiglass with the ground was increased at a rate of approximately 5 degrees per second. The angle at which the animal could no longer maintain its position and began to slide was recorded.
8. Escape from a Bowl—The hamster was placed in a seven inch deep metal bowl, and the number of attempts to escape from this bowl were counted over a 3 minute time period. An escape was counted each time the hamster grasped the rim of the bowl with its forepaws. Once an escape was counted, the hamster was placed back at the bottom of the bowl again.
9. Grasping Reflex—A wire loop was placed in the paw of the hamster. Success was determined if the hamster clung to the loop.
10. Righting Reflexes—These were a series of observations to determine the ability of the hamsters to assume a normal body orientation after they were placed in unnatural positions. Only success or failure was recorded, and time was not a factor.
    Body Flat—The hamster was placed flat on its back and success was determined if the animal rolled over to its feet.
    Body Tilted—The hamster was placed so that its front paws lay flat on a surface while the hind limbs of the animal were twisted counter-clockwise. Success was determined by whether or not the animal twisted its hind body for proper orientation.
    Hypnosis—The hamster was placed, paws up, in the palm of the hand. Success was determined if the hamster remained motionless (hypnotized) for 10 seconds.
    Dropped—The hamster was again taken in the palm of the hand, paws up, and then dropped 2–4 inches to the ground. Success was determined if the animal righted itself in the air and landed on its feet.
11. Placing Reactions—These were a series of observations which were performed on the hamsters to determine how they reached for a platform as they were brought near it while being held by the experimenter. Again, only success or failure was determined, and time was not a factor.

TABLE 1. (continued)

Forelimb—The hamster was held by the sides over a surface. Success was determined if the hamster attempted to touch the surface with its front paws.

Hindlimb—The hamster was placed on a surface in such a way that one of its hind legs hung over the edge. Success was determined if the animal attempted to place that hind leg on the surface.

12. Head Shaking—The experimenter lightly blew into the face of the hamster. Success was determined if the hamster reacted to the wind by shaking its head.

they could hang on the wire ($r = -0.58$, $F = 6.99$ w 1,14 df, $p < 0.05$), although their times were not significantly different from 12 month healthy hamsters. A different, U-shaped, pattern was seen when the time required to descend the pole was examined (Test 4, Figure 2B). In this test, 30 month healthy hamsters reached the ground very rapidly, while between 6 and 12 months of age, the time required to descend increased ($p < 0.05$, Dunn's). The results for the 30 month healthy hamsters occurred because these old hamsters no longer actually climbed down the pole, but instead fell down, i.e., 78% of the 30 month healthy hamsters fell whereas only 10 and 21% of the 12 and 6 month healthy hamsters fell (Chi-square = 11.66 w 1 df, $p < 0.01$). The time required to get to either the top or bottom of the inclined screen from the middle (Test 3, Figure 2C) was similar in 12 and 30 month healthy hamsters, as were the number of turns during escape from a container (Test 6c, Figure 2D).

CM hamsters showed none of the aging effects that healthy hamsters displayed. Figure 2A shows this most clearly. Six month hamsters of both strains held onto the horizontal

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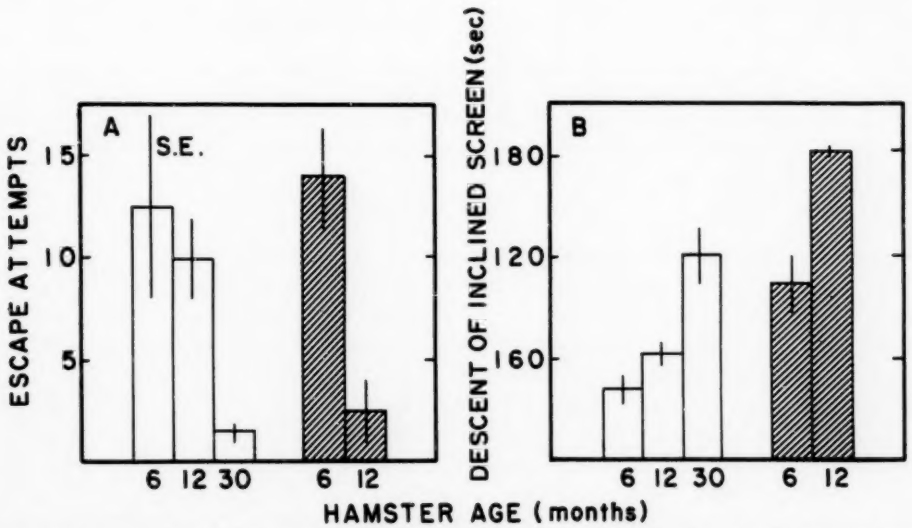

FIG. 1. Performance on two behavioral tests affected by age and disease: (A) Number of escape attempts from a bowl in 3 minutes (Test 8); (B) time to descend an inclined screen (Test 2). Performance on both tests showed monotonic changes with increasing age in healthy hamsters (open bars in all figures), while parallel changes occurred at an earlier age (12 months) in CM hamsters (hatched bars in all figures). $N = 6$ in 6 and 12 month groups, and N = 4 in 30 month group.

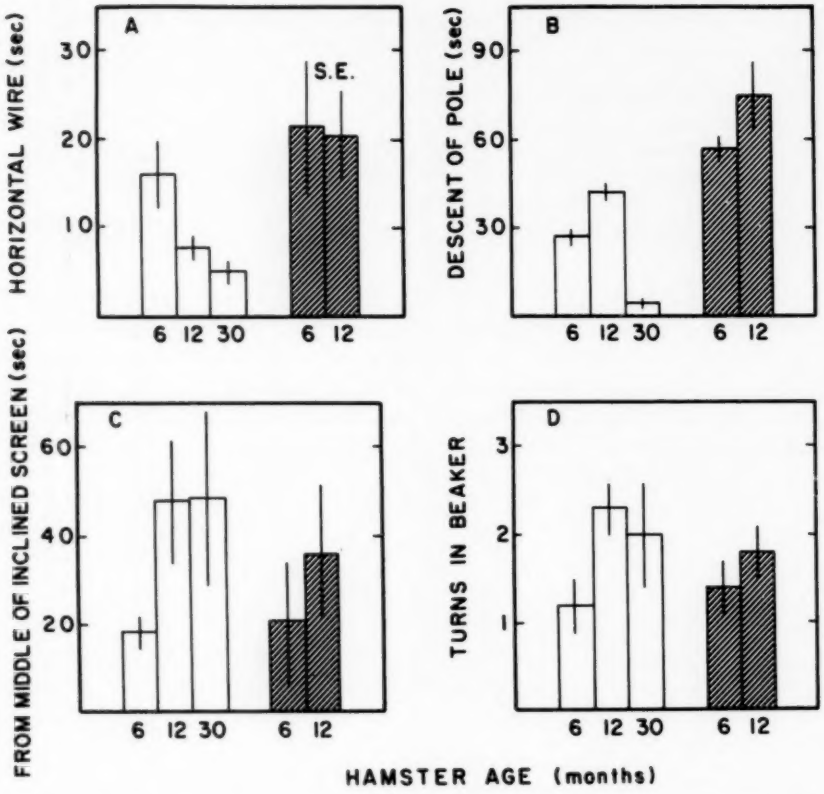

FIG. 2. Behavioral tests for which disease appeared to prevent age-associated changes: (A) Time hamsters held onto a horizontal wire (Test 1); (B) time to descend screen-covered, vertical pole (Test 4); (C) time to move to the top or bottom of inclined screen from the middle (Test 3); (D) number of turns during escape from an inverted beaker (Test 6c). In all tests, changes in performance between 6 and 12 months in healthy hamsters were not seen in CM hamsters.

wire for the same length of time and 12 month CM hamsters held on for a similar length of time, in contrast to 12 month healthy hamsters which fell significantly sooner than 12 month CM hamsters ($p < 0.05$, Dunn's). Similarly, in the other three tests shown in Figure 2, 6 and 12 month CM hamsters showed no significant differences in performance.

Figure 3 presents the results of a test in which no aging effect was seen in the healthy hamsters, but performance declined in the sick (12 month) CM hamsters. Thus, this test isolated an effect of disease on performance which was unrelated to changes due to normal aging. Healthy hamsters reared between 3 and 4 times during this test (6b) regardless of age, as did 6 month CM hamsters. However, 12 month sick CM hamsters reared less than either 6 month CM hamsters or 12 month healthy hamsters ($p < 0.05$, Dunn's).

Finally, strain effects, but neither aging or disease effects, were seen on the remaining tests. Thus, CM hamsters went up the inclined screen (Test 3b) more often than healthy controls (Chi-square = 13.46 w 1 df, $p < 0.005$), fell less often from the elevated platform (Test 5b; Chi-square = 6.29 w 1 df, $p < 0.01$), were able to maintain their position on the

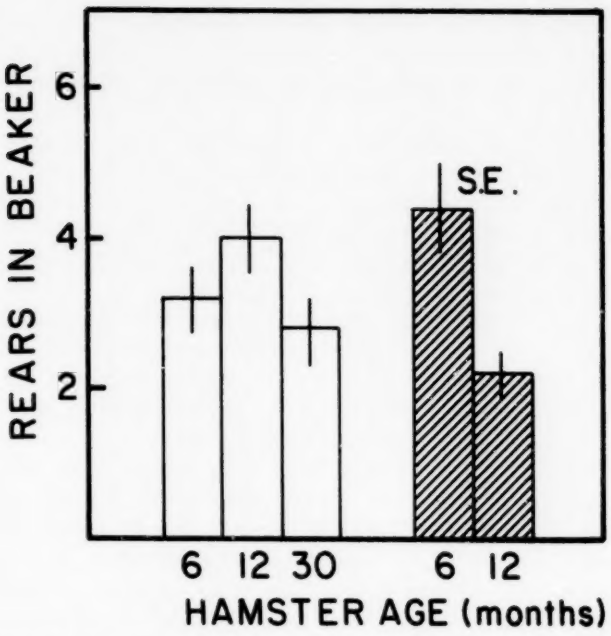

FIG. 3. Behavioral test affected by disease and not age: Number of rears during escape from an inverted beaker (Test 6b). Performance declined in 12 month CM hamsters, whereas it was constant from 6 to 30 months in healthy controls.

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Plexiglass slide at as great an angle (Test 7; F = 12.04 w 1,20 df, $p < 0.005$), and escaped from the container (Test 6) less often than healthy hamsters (Chi-square = 11.64 w 1 df, $p < 0.01$). Some strain effects may be due to the control hamsters being heavier than CM hamsters, but body weight was constant across the ages studied in each strain.

DISCUSSION

Our purpose in doing this work was to try to find performance tasks which would allow us to separate the effects of aging from effects related to disease. To do this we studied a strain of healthy hamsters at three times during their lifespan and a strain of cardiomypopathic hamsters at a point in their life when they appeared healthy and later in their life when they showed the puffy appearance and signs of decreased grooming consistent with the progression of their heart disease. Consonant with the reports of others studying aging in rodents, we found in this study of hamsters several tests which tracked chronological age. Importantly, this aging effect occurred in CM hamsters as well as healthy hamsters. The effect is seen most cleanly in Figure 1A which depicts the number of escape attempts made by hamsters placed in an open bowl. It was not until 30 months of age that healthy hamsters made fewer escape attempts than younger healthy controls. Six month CM hamsters made a similar number of escape attempts as 6 month healthy hamsters, which indicated no strain effects on the performance of this test. But 12 month CM hamsters made significantly fewer escape attempts than 6 month CM hamsters, in contrast to healthy 12 month hamsters which attempted the same number of escapes as the

6 month healthy hamsters. This earlier decline in performance of CM hamsters suggested an accelerated rate of aging as compared to what is seen in normal hamsters, an interaction between aging and disease, and specifically that disease in the CM hamster had aged it prematurely.

The test in which hamsters descended an inclined plane showed similar results. As seen in Figure 1B, normal hamsters took progressively longer to descend the screen as they got older. This effect was exaggerated in the CM hamsters with the 12 month CM hamsters not descending at all. Although a strain effect was also present, the fact that significantly longer times were not seen in healthy hamsters until they were 30 months of age while a similar slowing occurred in 12 month CM hamsters indicated a possible interaction between aging and disease. Thus, these two tests suggest that the biological age of CM hamsters is advanced at least to that seen in 30 month healthy hamsters. The inference that one would tentatively draw from data such as these is that a disease process may interact with the normal aging process to accelerate it, although it is possible that this inference may be limited to heart disease in CM hamsters.

What we would like to stress more than the above inference, which will require further evidence for substantiation, is the fact that the experimental strategy we have followed provides us data that even allows such an inference to be made. By developing a rate function of aging on performance, we can conclude that the rate of aging is speeded up in CM hamsters and that they are biologically older than 12 month healthy hamsters. In contrast to this strategy, others have compared healthier controls to groups of patients who either felt unwell (Webster and Logie, 1976), had hypertension (Furukama *et al.*, 1975), or died soon after evaluation (Borkan and Norris, 1980). In all of these studies, the biological age of the study group was significantly older than that of the healthy group. But this simple comparison at only one point in time does not deconvolve the interaction between aging and disease as clearly as understanding the rate of change in performance over time.

In addition to these aging effects, several tests seemed sensitive to disease but in qualitatively different ways (Figures 2 and 3). In some tests, a normal aging effect was lost when the CM hamster was studied over its lifespan (Figure 2). On the other hand, a diminution in rearing behavior was seen in the older CM hamsters but not in either of the older healthy hamster groups (Figure 3). Importantly, in this test, the performance of 6 month CM hamsters was not significantly different from that of 6 month control hamsters. Thus, we found tests that separate CM hamsters from healthy hamsters, but only when CM hamsters are sick. Similar to results for tests of aging, performance on these tests was the same for 6 month CM and healthy hamsters but not for older hamsters. The fact that both healthy and CM hamsters have similar baseline performance when young supports the proposition that the differences seen over time do not reflect a strain difference, but instead suggest that disease hastens the aging process in sick animals. The fact that such differences exist between performance in old and sick hamsters suggests that underlying neuro-behavioral mechanisms can be differentially affected by these two processes.

The other advantage to having these tests is they allow us to make a start at quantifying the effects of disease on tests that are commonly used as aging markers. Only in this way, have we been able to try to insure that the aging effects we measured in non-diseased animals were not due to some general debilitation resulting from unrecognized disease. Thus, rather than infer that disease is worse or better in any particular group under study, we can quantify its existence using tests such as these. This sort of approach is necessary if

we are to try to get away from the circularities inherent in our current thinking about aging and disease.

The interpretation posited to this point allows us to use test results derived from a normal population over its lifespan to learn about the aging process in a population bearing disease. In addition, having tests of aging and disease available may be useful in understanding whether the development of illness alters the aging process. For example, one view of aging is rather binary; the organism is healthy for a prolonged length of time, its time runs out, and then the organism becomes sick and soon thereafter dies (Shock, 1979). Having tests that might assess both aging and disease independently would allow us to collect data regarding this hypothesis. If the hypothesis were true, one would expect to find progressive aging effects as chronological age increases and then shortly before death, one would expect a shift to performance characteristic of the existence of disease (i.e., fewer rears in the beaker; longer times hanging from the horizontal wire).

In conclusion, aging populations may or may not harbor co-existing disease, and disease may or may not interact with the aging process to shorten normal lifespan. Nonetheless, efforts must be made to operationally separate the effects of one from the other. Our strategy has been to try to do this by comparing a healthy population to a sick population in which the chronological course of disease is well understood. Our results suggest a strategy that could prove useful in separating disease effects from those due to aging and that this strategy might fit well with others that are currently being used to understand what aging really is.

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AGE-ASSOCIATED CHANGES IN THE CIRCADIAN RHYTHM OF RETINAL N-ACETYLSEROTONIN AND MELATONIN IN RATS WITH PIGMENTED EYES

OLGA PULIDO and JONATHAN CLIFFORD

Department of Neurosciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

Abstract—Using a semiquantitative immunohistochemical procedure, we have evaluated the 24 hour circadian rhythm of *N*-acetylserotonin- and melatonin-like immunoreactivity in the retina of male hooded rats that have pigmented eyes. Three and twenty months old Long Evans rats were used. Animals were kept under a 14 light (*L*): 10 dark (*D*) lighting cycle and were sacrificed during the month of June. Four time points were assessed in the 24 hour light/dark cycle. Retinal *N*-acetylserotonin and melatonin were assessed by immunohistochemistry and microphotometric procedures as previously used by us. In young animals, the intensity of retinal *N*-acetylserotonin and melatonin immunofluorescence was significantly different ($P < 0.005$, $DF = 17$) in animals killed during the light vs the dark period with peak values during the dark period. In contrast, retinal *N*-acetylserotonin and melatonin-immunofluorescence in old animals showed a flattened 24 hour rhythm, with light values as high as those observed during the dark period. The age-associated bluntness of the 24 hour rhythm was more noticeable for *N*-acetylserotonin than for melatonin immunofluorescence.

Key Words: melatonin, *N*-acetylserotonin, indole, retina, aging, circadian rhythm, immunohistochemistry

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INTRODUCTION

MELATONIN IS present in the retina of several species, including man (Brecha, 1983; Bubenik and Purtill, 1980; Vivien-Roels *et al.*, 1981; Osol and Schwartz, 1984; Leino, 1984). Its presence in retina has been identified using a variety of immunochemical and biochemical procedures (Brown *et al.*, 1983a; Pang *et al.*, 1980; Leino, 1984; Osol and Schwartz, 1984). *N*-acetylserotonin, the immediate precursor of melatonin, is also present in rat retina, as demonstrated by radioimmunoassay (Pang *et al.*, 1981) and immunohistochemistry (Pulido and Clifford, 1985a,b). Retinal *N*-acetylserotonin and melatonin both exhibit a diurnal circadian rhythm (Pang *et al.*, 1980, 1981; Hann and Menaker, 1980; Grota, Holloway, and Brown, 1982; Pulido and Clifford, 1985a). In mammals, peak levels are observed during the dark phase (Yu *et al.*, 1981; Pang *et al.*, 1981; Pulido and Clifford, 1985a). Moreover, the enzymes involved in the synthesis of *N*-acetylserotonin from serotonin (*N*-acetyltransferase) and of melatonin from *N*-acetylserotonin (hydroxyindole-O-methyltransferase) are present in retina having a circadian rhythm (Binkley *et*

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al., 1979; Cardinali and Wurtman, 1972a; Hann and Menaker, 1980). The role of *N*-acetylserotonin and/or melatonin in retinal physiology is not known. Evidence indicates that it may play an important role in eye function (Besharse and Dunis, 1983; Bubenik *et al.*, 1978; Bubenik and Purtill, 1980). Melatonin has been shown to induce aggregation of pigment in the pigmented epithelium (Pang and Yew, 1979), to activate rod disk shedding (Besharse and Dunis, 1983), to increase eye weight and intraocular fluid content (Quay, 1984), as well as to inhibit adenylate cyclase (Niles, 1984) and dopamine release in the retina (Dubocovich, 1983). Less is known about *N*-acetylserotonin functions in retina. It appears to have less potent inhibitory action on dopamine release (Dubocovich, 1983), and does not have an effect on disk shedding (Besharse and Dunis, 1983).

Presently available evidence suggests that melatonin and/or *N*-acetylserotonin in the retina may play local roles on eye physiology, or systemic roles by participating in the entrainment of biological rhythms cued to light, particularly since the retina is a major component of the retino-hypothalamic-pineal axis. In this study we have investigated the effect of age, time of the day and lighting conditions on retinal *N*-acetylserotonin and melatonin as measured by semiquantitative immunohistofluorescence.

MATERIALS AND METHODS

Animals

Three and twenty month old Long Evans hooded male rats were kept under a 14 light (*L*): 10 dark (*D*) cycle using a full spectrum fluorescent light (Vita-Lite) which provided the luminance of a 120 foot candle at source (ceiling). Food and water were provided ad libitum. Animals were killed by decapitation during the month of June, at the following times: 4 or 10 hours after lights were on; 3 or 7 hours after the lights were off. Four to five animals were killed in each group at each time point. The entire eyeballs were rapidly enucleated, frozen in dry ice and kept at −70 °C until sectioned. A red bulb was used for illumination when animals were killed during the dark period.

Immunohistochemistry

Serial sections (15 μm) from the posterior segment of the eye were prepared in a cryostat and processed for *N*-acetylserotonin and melatonin immunohistochemistry using the same procedure previously described in detail (Brown *et al.*, 1983b; Pulido *et al.*, 1983a).

Tissue sections were incubated at room temperature for one hour with the first antiserum and for 30 minutes with the second antiserum. All antisera and normal rabbit serum (NRS) control were diluted 1:10 in phosphate buffered saline (PBS) pH 7.2. Three washes with PBS were done after each incubation. Slides were mounted in 50% glycerol: PBS pH 7.9.

Validation and Cross-Reactivity Studies

Experiments were carried out to investigate the specificity of each antisera as applied to immunohistochemistry in the retina of aged hooded rats with pigmented eyes. Samples of 200 μl of *N*-acetylserotonin and melatonin antisera (diluted 1:10) were individually incubated in a test tube, for two hours at room temperature, with 100 μg of each of the following substances: *N*-acetylserotonin; melatonin; serotonin (5-HT) and 5-methoxytryptophol. Approximately 80 μl of each mixture was placed on consecutive serial sections and slides were then incubated for one hour, followed by three rinses with PBS and by 30 minute incubation with the second antiserum.

Reagents

N-acetylserotonin (*R*-197) and melatonin (*R*-139) antisera were kindly donated by CIDtech Research Inc., 32 James St. S., Suite 301, Hamilton, Ontario L8P 2Y1 Canada. FITC conjugated anti-rabbit IgG was obtained from Daymar Laboratories (Toronto, Ontario). *N*-acetylserotonin (*N*-acetyl-5-hydroxytryptamine), melatonin (*N*-acetyl-5-methoxytryptamine), serotonin (5-hydroxytryptamine), and 5-methoxytryptophol were purchased from Sigma Chemical Co.

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Microphotometric Procedure

The procedure used has been previously described in detail (Pulido *et al.*, 1983b; Brown *et al.*, 1983b). Slides were examined in an incident light fluorescence microscope equipped with OSRAM HBO 100 W/Z mercury lamp, FITC 490 nm and K 510 nm suppressor filters, 4 nm BG exciting filter, and a photomultiplier photometer (Photovolt 520 *M*, Wild Leitz) (Pulido *et al.*, 1983a,b). Results are given in units corresponding to $\times 100$, each scale division represents 0.0001 foot candle (Pulido *et al.*, 1983b).

After stabilization and standardization of the equipment each set of slides were assessed at the same time. An eye from a young and an old animal at each time point was always processed and assessed simultaneously. A set of slides from each eye included samples incubated with melatonin antiserum, *N*-acetylserotonin antiserum, normal rabbit serum, phosphate buffered saline pH 7.2 and melatonin or *N*-acetylserotonin antiserum preabsorbed with *N*-acetylserotonin or melatonin. Several eyes from old animals were also tested for cross-reactivity with 5-HT or 5-methoxytryptophol. All readings were completed within the same day of processing the slides. An integrated measurement of the whole field of view was assessed using a 25X objective and 10X eye piece, which gives an overall magnification of 250X. A total of at least nine readings were recorded per slide and each slide had 2–3 sections. Eight to ten retinas were used for each parameter assessed. The arithmetic mean of the value obtained with *N*-acetylserotonin or melatonin antisera after subtraction of the normal rabbit serum background was the value assigned to each eye tested. The final data were analyzed using two-way analysis of variance (ANOVA) for age $\times$ time interaction and one-way ANOVA across time.

RESULTS

N-*acetylserotonin- and melatonin-like immunoreactivity*

The specificity of each antiserum was assessed as it relates to immunohistochemistry in the retina of aged hooded rats. Inhibition and cross reactivity studies gave similar results as those reported in young animals (Pulido and Clifford, 1985a), namely, selective preabsorption of *N*-acetylserotonin or melatonin antisera by *N*-acetylserotonin or melatonin, respectively. Addition of 5-HT to *N*-acetylserotonin or melatonin antisera did not alter the intensity of the immunofluorescence, but preabsorption with 5-methoxytryptophol induced a partial (20–30%) inhibition of both *N*-acetylserotonin and melatonin immunoreactivity (Pulido and Clifford, 1985a).

Retinas from older animals showed a higher background fluorescence as assessed by exposing the slides to normal rabbit serum. However, the normal rabbit serum values were not significantly different in old vs young rats. For quantitation studies, this background fluorescence was always subtracted from those obtained with the specific antisera.

In retinas of old animals, *N*-acetylserotonin and melatonin immunoreactivity were both visualized as a fluorescent stripe within the area corresponding to the inner segment of the photoreceptor cells and adjacent portion of the outer nuclear layer as well as the inner nuclear layer (Figures 1 and 2). Retinal *N*-acetylserotonin and melatonin topographic distribution in old animals was similar to that of young rats, namely photoceceptor layer, outer nuclear layer, and inner nuclear layer, with the highest fluorescent intensity observed within the junction between photoreceptor and outer nuclear layer (Pulido and Clifford, 1985a).

Diurnal variation of N-*acetylserotonin and melatonin immunoreactivity in retina from young and old animals*

In retinas from young animals, both *N*-acetylserotonin and melatonin showed significant 24 hour circadian rhythm (Figures 3 and 4), with a 60% increase in fluorescence intensity during the dark phase as compared to light values ($P < 0.05$).

In aged animals no significant difference is observed between the values of retinal *N*-acetylserotonin or melatonin immunofluorescence of animals killed during the dark

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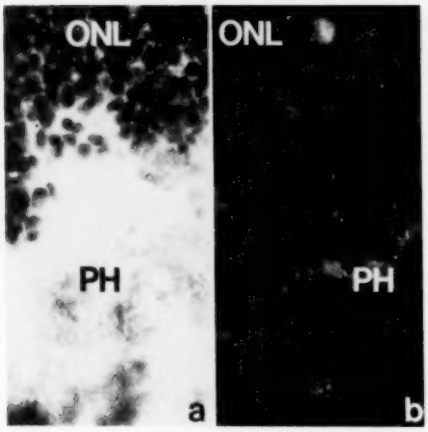

FIG. 1. Sections of retina from a 20-month-old rat killed during the dark period, showing a stripe of immunoreactive melatonin within the inner segment of the photoreceptor layer (PH), and the outer portion of the outer nuclear layer (ONL). Magnification in (a) 283X, as compared to normal rabbit serum control, (b) 283X.

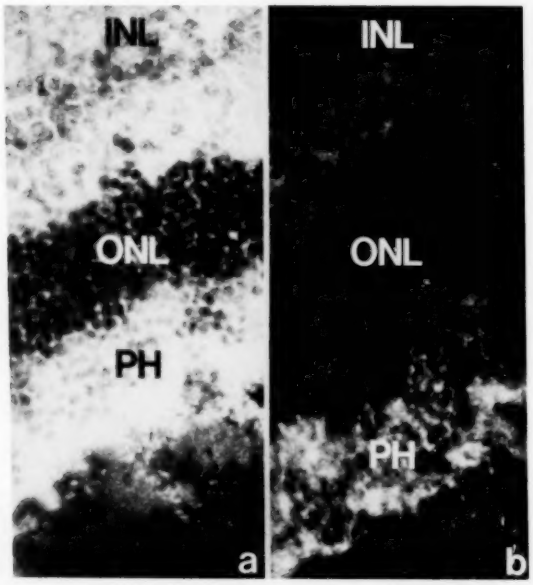

FIG. 2. Sections of retina from a 20-month-old rat killed during the dark period showing fluorescent stripes of immunoreactive *N*-acetylserotonin within the inner segment of the photoreceptor layer (PH), the outer portion of the outer nuclear layer (ONL), and the inner nuclear layer (INL). (a) 249X, as compared to normal rabbit serum control, (b) 249X.

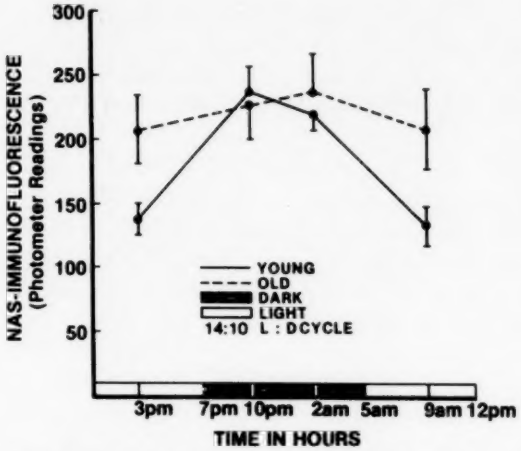

FIG. 3. *N*-acetylserotonin (NAS) immunoreactivity in retina of young (3-month-old) and old (20-month-old) rats. Values were obtained by subtracting background (NRS) measurements from NAS-antiserum readings. Results are expressed as the mean +/− SEM. Data were analyzed by two-way analysis of variance (ANOVA) for time and age as well as one-way ANOVA across time for each age group. Time × age effect at 3 p.m. shows a significance of $P < 0.05$, $DF = 7$.

period vs. those killed during the light period. In these animals the intensity of melatonin immunofluorescence during the dark phase was within the same range of values than those obtained in young animals killed at the same times. In contrast, aged animals killed at dark tend to have *N*-acetylserotonin and melatonin values higher than young animals killed at the same times. These changes were more prominent for *N*-acetylserotonin than

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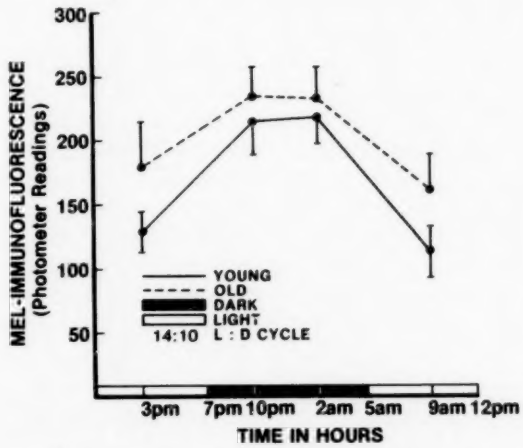

FIG. 4. Melatonin immunoreactivity in retina of young (3-month-old) and old (20-month-old) rats. Values were obtained by subtracting background (NRS) measurements from melatonin antiserum readings. Results are expressed as the mean +/− SEM. Data were analyzed by two-way analysis of variance (ANOVA) for time × age and by one-way ANOVA across time for each age group.

melatonin. The time and age effect was significant ($P < 0.05$) for *N*-acetylserotonin in retinas of animals killed at 3 p.m. Although not significantly different, light values of *N*-acetylserotonin at 9 a.m. and light values of melatonin immunofluorescence at 9 a.m. and 3 p.m. were 30–50% higher than those obtained in retinas of young animals.

DISCUSSION

Evidence indicates that melatonin, *N*-acetylserotonin and other indole derivatives are present in retinas of several species (Baleman *et al.*, 1980; Bubenik *et al.*, 1978; Pang *et al.*, 1980, 1981; Reppert and Sagar, 1983). Melatonin has been shown to be present in the human retina (Leino, 1984; Osol and Schwartz, 1984). The function of melatonin and other indole compounds in the retina is not yet known. Retinal *N*-acetylserotonin and/or melatonin might play a role in the synchronization of biological rhythms cued to light by regulating photic messages which will then travel through the retino-hypothalamic-pineal pathway (Cardinali, 1983; Reiter, 1983; Moore, 1973). Evidence indicates that the anatomical connections between the retina and hypothalamus are also present in humans (Sadun *et al.*, 1984). Therefore, it is likely that in man, as in other mammals, the diurnal light–dark cycle acts to entrain the endogenous circadian rhythms. One of the most significant aspects of indole synthesis in the pineal as well as the retina is that it follows a circadian rhythm (Binkley *et al.*, 1979; Gern and Ralph, 1979; Cardinali, 1983). We found that in young animals the intensity of retinal *N*-acetylserotonin and melatonin immunofluorescence increases 60% during the dark as compared to the light. This data agrees with that reported by other investigators using other techniques and animal species (Pang *et al.*, 1980, 1981; Yu *et al.*, 1982). Radioimmunoassay measurements of retinal *N*-acetylserotonin and melatonin content as well as the release of these compounds in an in vitro preparation show circadian rhythms with peak values observed during the dark phase (Pang *et al.*, 1980, 1981; Yu *et al.*, 1982). There is also evidence that in the retina the activity of the enzyme *N*-acetyltransferase, which converts serotonin to *N*-acetylserotonin, responds to environmental lighting, both in vitro and in vivo with the highest activity during the dark phase (Binkley *et al*, 1979).

All these studies on retinal *N*-acetylserotonin and/or melatonin and their synthesizing enzymes have been carried out using young adult animals. We now show that in contrast to young animals, in aged Long Evans hooded rats the intensity of retinal *N*-acetylserotonin and melatonin immunofluorescence was not significantly different during light vs. dark, with light values as high as those seen during the dark. A significant ($P < 0.05$) time and age effect was observed on *N*-acetylserotonin immunoreactivity at 3 p.m., during the light phase. Although, at other times *N*-acetylserotonin and melatonin immunoreactivity was not significantly different in old vs. young animals, the light values always tend to be higher (30–50%) in aged as compared to young animals killed at the same times (9 a.m., 3 p.m.). The data reported here were obtained using a semi-quantitative method and awaits corroboration by a biochemical quantitative procedure. Despite the limitations of the methodology, these data suggest that there is an age-associated effect on retinal *N*-acetylserotonin and melatonin circadian rhythm, with a flattened 24 hour rhythm. It is possible that with increasing age the biosynthetic pathway of *N*-acetylserotonin and melatonin become less responsive to the inhibitory effect of light leading to higher light values. Other photomechanical factors intrinsic to the eye may also be altered. Further investigations are required to differentiate changes in retinal response *per se*, versus visual changes leading to a decrease in light reaching the retina.

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Animals used in this study were also assessed for pineal and serum melatonin levels as measured by radioimmunoassay (Pulido *et al.*, 1985b). We found a significant age-associated reduction on the nocturnal–pineal and circulating melatonin levels (Pulido *et al.*, 1985b). High *N*-acetylserotonin and melatonin immunofluorescence values in aged animals during the light phase might then represent a compensatory response to an age-associated reduction in pineal and circulating melatonin levels, as suggested by Yu *et al.* (1981), in long-term pinealectomized rats. Keeping with this hypothesis, Cardinali *et al.* (1972b) have shown that exposure of rats to a continuous green light for 17 days resulted in almost total loss of pineal hydroxyindole-0-methyltransferase activity, whereas in the retina there was a significant stimulation of the activity.

Retinal melatonin and *N*-acetylserotonin may also play an important role in local eye physiology. At least for melatonin there is evidence indicating that it may modulate eye pigmentation (Pang and Yu, 1979), photoreceptor metabolism (Besharse and Dunis, 1983), dopamine release (Dubocovich, 1983), and adenyl cyclase activity in the retina (Niles, 1984). Melatonin and other methoxyindoles have been shown to activate rod shedding in vitro (Besharse and Dunis, 1983). Melatonin administration appears to induce an increase in volume of intraocular fluid, as well eye and lens weight (Quay, 1984). These effects were particularly prominent with large doses of melatonin. Although further investigations are required in this regard, this data suggests that melatonin and maybe other indoles can modify several aspects of eye function. Bubenik and Purtill (1980) suggest that dopamine and melatonin modulate the susceptibility of the retina to damage induced by continuous high intensity illumination, with dopamine playing a protective role. On the other hand, Dubocovich (1983) has shown that melatonin as well *N*-acetylserotonin are potent inhibitors of dopamine release in retina. Hence, age-associated changes on retinal *N*-acetylserotonin and melatonin might be important in the susceptibility to light-induced retinal damage.

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It is also possible that age related changes on retinal *N*-acetylserotonin and melatonin may be important factors underlying age-related retinal changes such as those reported on the pigmented epithelium of pigmented rats (Katz and Robison, 1984). To this effect, melatonin has been shown to induce aggregation of pigment in the pigmented epithelium (Pang and Yew, 1979).

In conclusion, knowledge of the function of melatonin, *N*-acetylserotonin, and other indoles within the eye, as well as of their mechanisms of synthesis and regulation are at present still fragmentary and incomplete. However, evidence suggests that melatonin and *N*-acetylserotonin may play important roles in several aspects of eye physiology as well as in synchronization of biological rhythms to light by modulating the retino-hypothalamic-pineal axis. In keeping with this hypothesis, we have recently demonstrated age-related increase in the population of (3H) melatonin binding site in rat hypothalamus of animals killed during the dark period which directly correlates to a decrease in the nocturnal levels of circulating melatonin (Niles and Pulido, 1985). In addition, we present here evidence of age-related changes in retinal circadian rhythms of *N*-acetylserotonin and melatonin immunoreactivity. Retinal *N*-acetylserotonin and melotonin in young animals have peak levels during the dark period (Pang *et al.*, 1980, 1981; Pulido and Clifford, 1985a). These circadian changes are probably due to a higher activity of the enzyme *N*-acetytransferase during dark vs. light (Binkley *et al.*, 1979; Harm and Manaker, 1980). Evidence suggests that in the retina as in the pineal gland the activity of this enzyme is modified by the environmental lighting conditions and may also be responsible for the rhythmic production

of *N*-acetylserotonin and melatonin in the retina, which is thought to be unique to the pineal gland (Birkley *et al.*, 1979; Harm and Manaker, 1980). It is therefore possible that age-related changes in retinal *N*-acetylserotonin and melatonin are related to decreased responsiveness of the activity of *N*-acetyltransferase to environmental lighting conditions. Studies are in progress to verify the above data by quantitative biochemistry and to assess several aspects of the synthesis and regulation of *N*-acetylserotonin and melatonin in the retina.

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AGE-RELATED CHANGES IN THE CORNEAL ENDOTHELIUM OF THE MOUSE

KENNETH L. FITCH and MATHEW J. NADAKAVUKAREN

Department of Biological Sciences, Illinois State University, Normal, Illinois 61761

Abstract—The endothelial cells of the C57BL/6J mouse cornea show a progressive age-related increase in cell area, and an increasing departure from the initial hexagonal form. Mean endothelial surface areas were 304, 386, 458, 499, and 653 μm^2 for mice of ages 1, 4, 11, 22, and 27 months, respectively. Increasing cell areas are interpreted to represent a spreading and thinning of surviving cells to compensate for cell loss. These age-related changes correspond to those previously observed in the rat, although the rat endothelial cells tend to have smaller cell areas and show less variation at each age studied.

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INTRODUCTION

THE MAMMALIAN corneal endothelium, which covers the posterior surface of the cornea, is a single layer of cells which are thin and inconspicuous in cross section, but which appear as a regular mosaic of hexagonal cells in flat preparations. Despite its thinness, the endothelium is an essential structure for the maintenance of normal deturgescence and transparency of the cornea (Waltman, 1981). The ability of the endothelium to regenerate is species specific. Mitotic activity following injury has been demonstrated in the cat and rabbit (Van Horn *et al.*, 1977). Gloor *et al.* (1980) demonstrated mitoses after scarring of the monkey cornea, but Van Horn and Hyndiuk (1975) concluded, on the basis of electron microscope studies, that healing of the primate endothelium is mostly by migration and enlargement of the cells. The human adult appears to have very little (Kaufman *et al.*, 1966) or no mitotic repair activity (Cogan, 1951; Doughman *et al.*, 1976). Damaged or lost endothelial cells in man are compensated for by spreading and thinning of neighboring cells (Bigar, 1982). Specific areas of endothelial cell absence have been documented in humans (McCarey and McNeill, 1977), and a variety of operative procedures have been shown to reduce the endothelial cell population (Polack and Sugar, 1976; Forstot *et al.*, 1977).

Laule *et al.* (1978), using the specular microscope, calculated that the total average cell population in the human corneal endothelium declines from 935,000 cells per cornea at age 3, to 650,000 cells at age 25, and 500,000 cells at age 60, while average cell density decreased from about 4400 cells per mm^2 at age 3 to approximately 2850 cells per mm^2 at age 25, and 2329 at age 61. Fitch *et al.* (1982), using scanning electron microscopy, showed mean endothelial cell areas of female Sprague-Dawley rats 6, 14, and 30 months old to be 251, 336, and 405 μm^2, respectively, which represent cell densities of 3891, 2976, and 2469 per mm^2. The latter is the only report, aside from human studies, on the corneal en-

dothelium of animals throughout the life span. We now report on a similar study of the mouse corneal endothelium.

MATERIALS AND METHODS

Twenty female C57BL/6J mice, four each from ages 1, 4, 11, 22, and 27 months, were obtained from the National Institute of Aging. The median and maximum survival of these mice are 128 and 183 weeks, respectively (Cheney *et al.*, 1980). The eyes of the mice were removed immediately after decapitation. The anterior one-half of each eye was excised and the central cornea cut into two segments less than 1 × 2 mm in a cold bath of phosphate buffered (pH 7.3) 5% glutaraldehyde. A 30-minute fixation in glutaraldehyde was followed by a 30-minute fixation in buffered 1% osmium tetroxide, dehydration in acetone and critical point drying using liquid carbon dioxide. The samples were then coated with a 20-nm thick layer of gold and examined in a Cambridge Stereoscan scanning electron microscope operating at 10 kV.

All samples were photographed at 1000X magnification. The portion to be photographed was randomly selected from each cornea. Endothelial cells from the center of the photographs were used for area calculations. Cell areas were determined by tracing the cell outlines on polaroid photographs, using a Zeiss Video Plan II Digitizer and Image Analysis System. Both eyes from four animals in each group were used in the study for a total of 50 cells from each age group. The data were statistically analyzed using the *t* test.

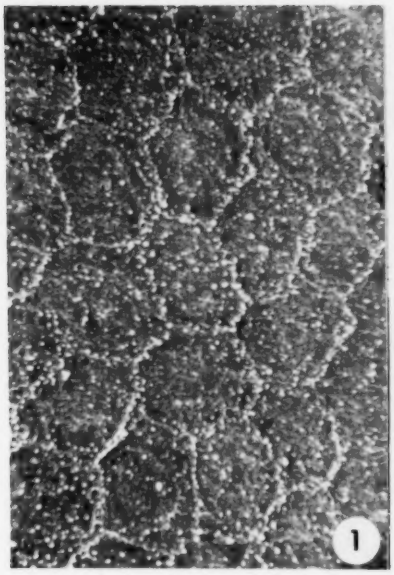
RESULTS AND DISCUSSION

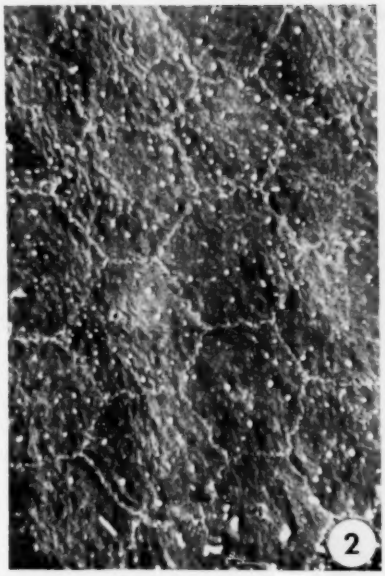
Figures 1–4 show scanning electron micrographs of central corneal endothelium of 1, 4, 11, and 27-month-old mice. The pattern of increasing cell size and irregularity of cell borders found in the human and Sprague-Dawley rat is also seen in the mouse eye. This is interpreted to represent a spreading and thinning of surviving cells to compensate for cell loss. The frequency distribution of microvilli appears unrelated to age, with some cells showing none and others few to many. This is similar to our observation of the corneal endothelium in the rat and is in contrast to the reports of 20–30 microvilli per cell in human (Hogan *et al.*, 1971) and rabbit (Blümcke and Morganroth, 1967). Occasionally we observed areas with deepened and highly irregular cell boundaries in the 27-month-old mouse. Such areas were also found, but with less frequency, at 22 months. Indistinct cell boundaries and areas of vacuolization, degeneration, and cellular disruption also were seen rarely in the 27-month-old mouse.

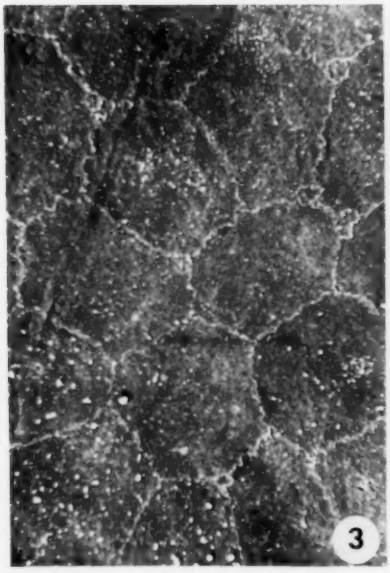
Figure 5 shows the changes in the frequency of endothelial cell areas as a function of age. Although the tendency toward cells of larger surface area with age is clearly evident, the range of cell areas was large within each age group, the range becoming increasingly larger with each age increment. This variation accounts for the lack of statistical significance between ages 11 and 22 months, even though a trend is apparent both in mean cell area and in maximum cell size measured.

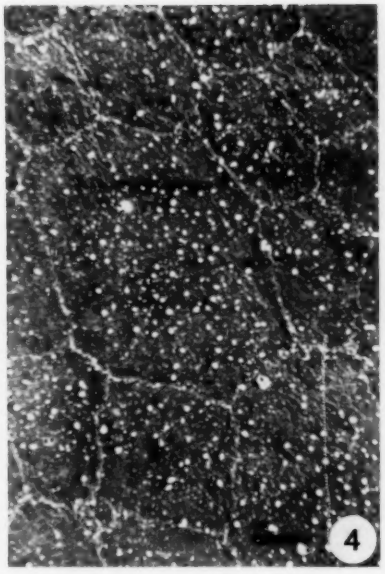
Although the enlargement of cell area of endothelial cells with age is not as clearly defined as we found in the Sprague-Dawley rat, the same process of cell loss is evident. The fact that we used only three ages of rats (6, 14, and 30 months) may account for the better statistical separation we obtained with the rat corneal endothelium. Mean cell areas in the rat endothelium in the three age groups were found to be 251, 336, and 405 μm^2, respectively. Means in our five ages of mice are 304, 386, 458, 499, and 653 μm^2 for ages 1, 4, 11, 22, and 27 months, respectively. Thus rat endothelial cells seem to have smaller cell areas and show less variation at each age studied.

The report of Schwartz *et al.* (1982) on corneal opacification and other corneal abnormalities in 9% of 400 animals of this mouse strain is consistent with our finding of greater pathology in the older age groups, pathology that was not observed in the rat corneas. However, corneal opacification was not evident in our series. They also reported









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FIGS. 1–4. Scanning electron micrographs of the corneal endothelium of female C57BL/J6 mice aged 1, 4, 11, and 27 months, respectively. Cell enlargement and distortions of the basic hexagonal form are evident with increasing age. White dots are microvilli whose occurrence appears unrelated to age. All micrographs are of the same magnification (marker represents 10 μm).

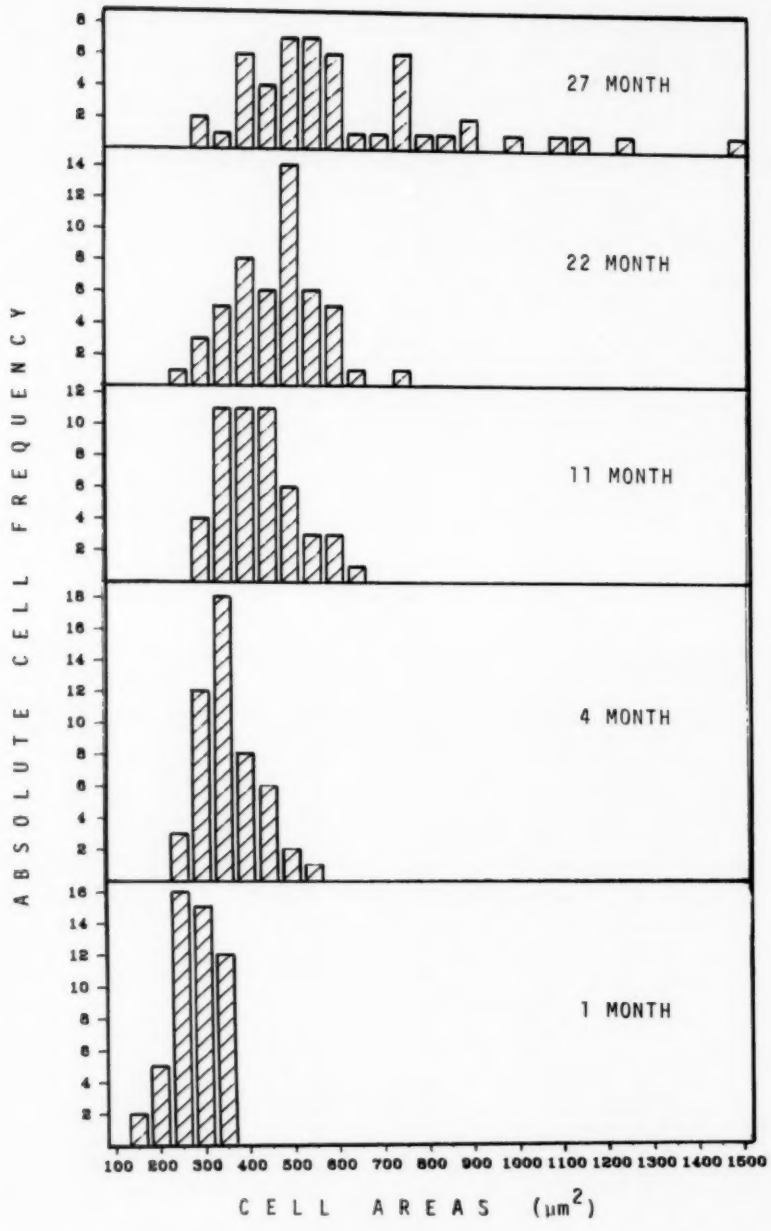

FIG. 5. Frequency distributions of corneal endothelial cell areas of five ages of female C57BL/J6 mice. t test revealed significant differences between mean cell areas of all age groups except that between 11 and 22 months. Statistical differences between ages 1, 4, 11, and 27 months were at the $p < 0.01$ level, and the differences between ages 22 and 27 months were at the $p < 0.02$ level.

that a majority of their animals showed anterior synechiae, and in a few cases almost complete adherence of the iris to the posterior surface of the cornea, thus obliterating the anterior chamber. These iridocorneal adhesions were also observed in a majority of our animals. It is possible that we are observing some pathological processes inherent in this strain, as described by Schwartz and co-workers, superimposed on the normal ontological loss and enlargement of endothelial cells. Nevertheless, our data suggest that the corneal endothelium can be useful as an index of age in gerontological studies of the mouse.

Acknowledgment—We thank Dr. Stephen Gates for providing the animals used in this study.

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MEMBRANE FLUIDIZATION INCREASES LOW-AFFINITY MUSCARINIC RECEPTOR BINDING IN BRAIN: CHANGES WITH AGING

GERHARD FREUND[1], THOMAS R. BROPHY III, and JAMES D. SCOTT

Veterans Administration Medical Center and Departments of Medicine and Neuroscience, Center for Alcohol Research, University of Florida, Gainesville, Florida

Abstract—Specific cholinergic muscarinic receptor binding was determined with L-[^{3}H] quinuclidinyl benzilate ([^{3}H]QNB) in homogenates from crude synaptosomal pellets prepared from mouse whole-brain homogenates. Specific total (high- and low-affinity) binding was determined in the absence of the agonist carbachol and low-affinity binding in its presence. These membrane preparations were fluidized by adding in vitro aliphatic alcohols ranging from ethanol to hexanol and by increasing the incubation temperatures. At 23 °C hexanol (14.7 m*M*) nearly doubled the low-affinity binding in the presence of carbachol (0.32 m*M*) and decreased high-affinity binding by the same amount. This suggested a change of muscarinic receptors from high- to low-affinity conformation. Increase of incubation temperature from 24 °C to 37 °C nearly tripled low-affinity binding.

Brain homogenates from female C57BL/6J mice, ages 6, 12, 18, and 30 months, showed a progressively lower stimulation by hexanol of low-affinity [^{3}H]QNB binding in the presence of carbachol. We postulate that this diminished change with age of [^{3}H]QNB-receptor binding in response to alcohols may be a result of increasing membrane rigidity with advancing age. Rigidity of membranes may link aging at the membrane level, synaptic receptors, and impaired learning behavior.

Key Words: muscarinic receptor binding, membrane fluidization, mouse brain, aging of brain receptors

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INTRODUCTION

AGING AND chronic alcohol consumption impair learning and memory in humans and animals (Freund, 1980, 1982a,b; Kubanis *et al.*, 1982). The causes of these deficits could range on a continuum from loss of entire cells, their processes, and synapses to purely molecular changes in synapses. The common terminal pathway is impaired or lost synaptic function (Bartus *et al.*, 1982; Samorajski, 1981). Aging and alcohol could affect transmitter production, receptor numbers and affinity, membrane environment of receptors, postsynaptic coupling, or effector events, or a combination of these. One of the factors that is thought to influence and restrict receptor binding is the lipid membrane environment of the receptors (Birdsall *et al.*, 1983).

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[1]Correspondence and requests for reprints should be addressed to Gerhard Freund, M.D., Medical Service (111), Veterans Administration Medical Center, Gainesville, FL 32602.
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There is evidence that increased membrane lipid microviscosity in older mice is associated with a decrease of transmitter-receptor binding (Samuel *et al.*, 1982). However, many changes associated with aging have no significant effect on function. We therefore wanted to test two hypotheses. First, the changing of membrane fluidity with alcohols and incubation temperatures will alter receptor binding if the lipid membrane microenvironment is relevant to receptor binding. Second, if the age-associated increased membrane rigidity has a potential effect on receptor binding, then the older, rigid membranes should be more resistant to fluidization-induced receptor binding changes by the same concentration of an alcohol.

The purpose of this investigation was to determine how increasing membrane fluidity with aliphatic alcohols and higher incubation temperatures affect muscarinic cholinergic receptor binding in brain homogenates of old and young mice. Brain postsynaptic muscarinic receptors are thought to exist in at least two interconvertible functional and conformational states (Birdsall *et al.*, 1983). Briefly, the receptor that is not coupled to an effector (enzyme or ion channel) is in a "high-affinity" state that converts to a "low-affinity" state with coupling. Appropriate concentrations of muscarinic antagonists such as quinuclidinyl benzylate (QNB), because of a very high affinity for the receptor, occupy almost all of the available receptor binding sites irrespective of their degree of affinity. In contrast, agonist agents, like carbachol, have generally lower affinities for the receptor and therefore in high concentrations compete for and occupy mostly high-affinity receptor sites. The low-affinity agonist carbachol in appropriately high concentrations will block most high-affinity sites but will leave the low-affinity (presumably effector-coupled) receptor sites available for binding with low concentrations of high-affinity QNB. The QNB binding in the presence of carbachol reported here is therefore a measure of the number of "low-affinity" muscarinic receptor binding sites.

MATERIALS AND METHODS

Female C57BL/6J mice (age 2 months) were purchased and housed in animal facilities until they were 6, 12, 18, or 30 months old. The mice were fed Purina Laboratory Chow (Ralston Purina Company, St. Louis, MO) *ad libitum*. At the specified ages the mice were decapitated, the brains were removed, and the cerebellum and olfactory bulbs were separated. The brains were immediately homogenized with a Brinkman Polytron homogenizer at power setting 5 for 15 seconds in ice-cold 0.05-M sodium potassium-phosphate buffer (pH 7.4). The resulting concentration was approximately 50 mg tissue/ml buffer (5% w/v, 4.5 mg protein/ml buffer). Crude synaptosomal fractions were prepared by centrifugation at 50,000 × *g* for 10 minutes. The pellets were resuspended in the original volume of fresh Na-K buffer by the homogenizer at setting 5 for 5 seconds. This resulted in concentration of approximately 2.1 mg of 50,000 g resuspended pellet protein per milliliter of buffer. Individual incubates contained resuspended 50,000 × *g* homogenate, 50 μl/ml buffer.

Muscarinic cholinergic receptors were determined by the method of Yamamura and Snyder (1974) adapted as described previously (Freund, 1980). ^{3}[H]L-quinuclidinyl benzylate ([^{3}H]QNB), 40 Ci/mmol, was purchased from New England Nuclear Corporation, Boston, MA. [^{3}H]QNB was added in the concentration of 5.4×10^{-10} *M* to all tubes.

To assay specific binding, we incubated 100 μl of the homogenates for 1 hour at various temperatures with 2 ml of 0.05 *M* sodium-potassium (Na-K) phosphate buffer, pH 7.4, containing the [^{3}H]QNB. Nonspecific binding was determined in the presence of unlabeled 2.5 μ*M* QNB (Yamamura and Snyder, 1974), a gift from Hoffman La Roche, Nutley, NJ. Every determination was performed in triplicate. The incubation was terminated by vacuum filtration through glass-fiber filters (GF/B, Whatman). The filters were washed three times with 3 ml ice-cold buffer. The filters were transferred to liquid scintillation vials. Glacial acetic acid, 50 μl, and 0.5 ml Protosol (New England Nuclear) were added, and the mixture was heated in a water bath at 57 °C for 30 min. After the mixture was cool, 10 ml of Econofluor (New England Nuclear) were added; the vials were stirred and counted after 36 hours of adaptation to dark.

Carbamylcholine chloride (carbachol; Sigma, St. Louis, MO) was added to the incubated samples in concentration 0.32 m*M*, which suppressed specific QNB binding to approximately 30% of baseline (McKinney and Coyle, 1982). Hexylalcohol (hexanol; Eastman Kodak, Rochester, NY) was added in the concentration of 14.7

m*M*. The other alcohols were obtained from Fisher Scientific Co. Protein concentrations were determined in 50-μl aliquots from the resuspended pellet homogenates by the method of Lowry *et al.* (1951), using bovine albumen as standard. Specific binding was calculated as total minus nonspecific. The results were expressed as the meant ± SEM of the number of experiments indicated. Statistical comparisons were made using Student's *t* test (Dixon and Massey, 1969).

RESULTS

Aliphatic alcohols in vitro under appropriate conditions only diminished but did not increase specific high-affinity [^{3}H]QNB binding in resuspended 50,000 *g* pellets from mouse whole-brain homogenates (data not shown). However, low-affinity binding in the presence of carbachol was enhanced (Figure 1), most marked by hexanol, and least by ethanol in descending order with decreasing chain length. This enhancement increased with increasing concentrations of alcohol up to a certain concentration. Beyond an optimal concentration of the alcohols, low-affinity binding was diminished to well below baseline control levels. All values were determined in triplicate. Figure 1 is one representative of three individual curves that varied less than 6%.

Effect of incubation temperatures on specific, total (high- and low-affinity) QNB bind-

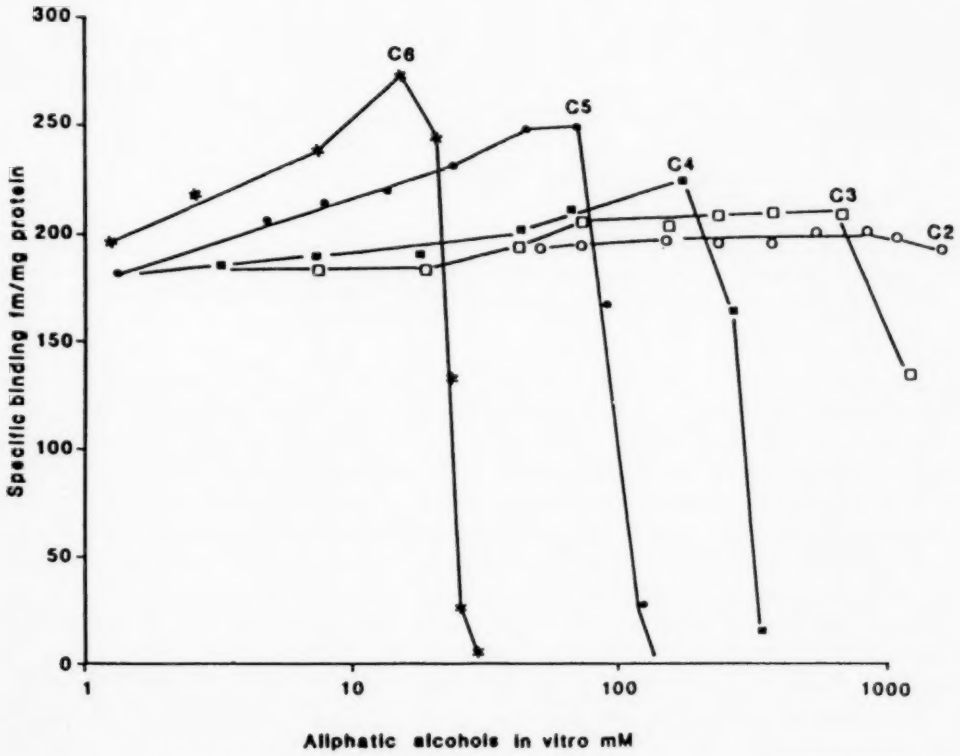

FIG. 1. The effect of various aliphatic alcohols (from C2-ethanol, C3-propranol to C6-hexanol) on specific, low-affinity [^{3}H]QNB (5.4×10^{-10} m*M*) binding in the presence of 0.32 m*M* carbachol (low-affinity binding) at 23 °C incubation temperature. Resuspended 50,000 × *g* pellets of mouse whole-brain homogenate (5% w/v).

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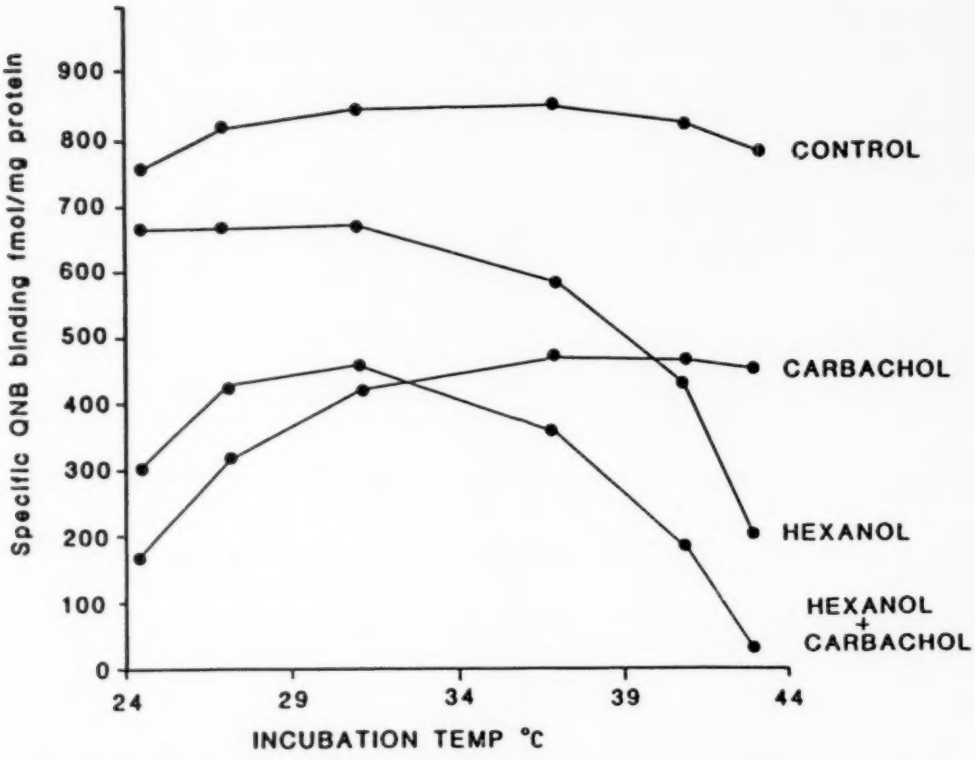

FIG. 2. The effect of incubation temperatures on specific QNB ([^{3}H]QNB 5.45 × 10^{-10}*M*) binding: (a) total (high- and low-affinity) without in vitro additions (CONTROL); (b) HEXANOL only 14.7 m*M*; (c) low-affinity binding in the presence of carbachol, 0.32 m*M*; and (d) low-affinity binding in the presence of both HEXANOL and CARBACHOL. Resuspended 50,000 × *g* pellets of mouse whole-brain homogenate (5% w/v).

ing is shown in Figure 2. (a) The CONTROL curve shows the effect of increasing the incubation temperature from 24 °C (room temperature) to 43 °C on [^{3}H]QNB binding. The changes are small (7%). Binding is maximal at 37 °C and declines slightly at higher and lower temperatures. (b) When hexanol is added to the incubate, the total (high- and low-affinity) binding at room temperature decreases. At higher temperatures binding decreases even further. (c) When carbachol is added to the homogenate, only low-affinity binding is determined. It is evident from Figure 2 that low-affinity binding at room temperature decreases. At higher temperatures binding decreases even further. (c) When carbachol is added to the homogenate, only low-affinity binding is determined. It is evident from Figure 2 that low-affinity binding at room temperature almost triples at body temperature and then slightly declines with even higher incubation temperatures. (d) Low-affinity binding in the presence of carbachol is enhanced also by hexanol at room temperature. Increasing the temperature to 29 °C further increases low-affinity binding. However, with a further increase of temperature in the presence of hexanol, low-affinity binding markedly declines, probably as a result of over-fluidization by the combined fluidization effects of hexanol and raised temperature. Generally, at room temperature,

hexanol increases the low-affinity binding (carbachol plus hexanol) to the same extent as it decreases the total (low- plus high-affinity) binding. This fact suggests that hexanol fluidization favors a change from a high- to a low-affinity conformation. This reciprocal relationship becomes even more pronounced at higher temperatures, up to approximately 30 °C. In contrast, hexanol combined with temperatures elevated beyond 30 °C, results in a decrease of all binding, probably because of overfluidization. Interestingly, with carbachol in the medium, raising the incubation temperature to 37 °C increased [^{3}H]QNB binding. However, when the membranes were fluidized even more by the further addition of hexanol, [^{3}H]QNB binding decreased. There was no significant effect on nonspecific binding (data not shown).

The effect of increasing age on specific [^{3}H]QNB binding is shown in Figure 3. As has been shown previously under different in vitro conditions, total (high- plus low-affinity)

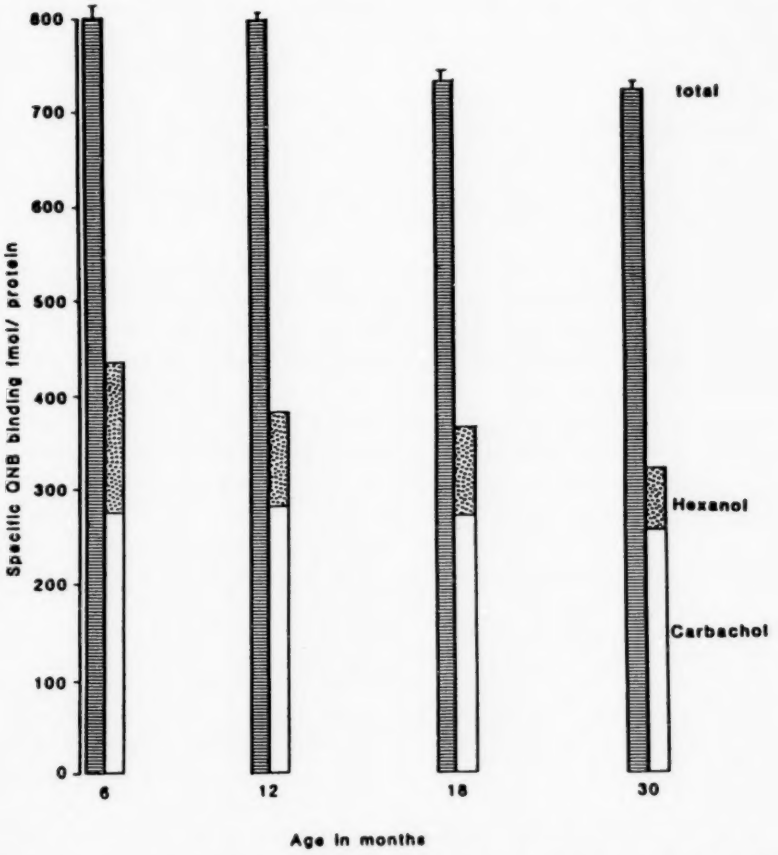

FIG. 3. Effect of age on specific [^{3}H]QNB binding. Total binding is high-affinity plus low-affinity binding. Low-affinity binding is determined in the presence of carbachol. Low-affinity binding is stimulated by hexanol. Mouse whole-brain homogenate, [^{3}H] QNB 5.45 × 10^{-10}*M*, carbachol 0.32 m*M*, hexanol 14.7 m*M*, incubation temperature, 23 °C, N = 8 per age group. The differences between hexanol-induced changes are significant ($p > 0.05$) by Student's *t* test.

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binding is decreased in mouse whole brain with advancing age (Freund, 1980; Kubanis *et al.*, 1982). In this experiment there is a decrease of 8.5% ($p < 0.01$) between the ages of 12 and 18 months. Low-affinity binding in the presence of carbachol is not significantly decreased with aging, but the magnitude of hexanol-induced increase of low-affinity binding diminishes progressively with age, from 431 ± 20 to 334 ± 7 fmol/mg protein. The results obtained with pentanol (57 m*M*) and butanol (162 m*M*) were very similar (data not shown).

DISCUSSION

The impairment of cognitive function associated with aging and prolonged alcohol consumption may be the result of morphologically recognizable loss of neurons or their component parts, or both. In addition, only chemically detectable losses, such as decreased synaptic receptor densities and affinities (Bartus, 1982; Freund, 1984), may impair learning and memory. Finally, aging and chronic alcohol consumption may affect synaptic membrane structure, which, in turn, may affect the binding of synaptic transmitters to their respective receptors. The presence of ethanol in neuronal membranes increases their fluidity and compensatorily changes their chemical structural composition (Chin and Goldstein, 1977; Ingram, 1982a,b; Seeman, 1972). These changes in membrane structure and fluidity could, in turn, influence receptor binding. The purpose of this investigation was to determine whether fluidization of membranes by aliphatic alcohols and elevated incubation temperatures could alter receptor binding. We also wished to learn whether such changes were affected by age.

We incubated crude synaptomal fractions obtained from mouse whole-brain homogenates at room temperature with the muscarinic cholinergic antagonist [^{3}H]-QNB. The addition in vitro of aliphatic alcohols decreased the total specific (high- plus low-affinity) binding and increased proportionately the low-affinity binding determined in the presence of the unlabeled agonist carbachol. This could be interpreted as a direct effect of alcohols on the receptor molecules by changing them from a high- to a low-affinity conformation (Birdsall *et al.*, 1978, 1980; McKinney and Coyle, 1982). Alternatively, this change could be induced indirectly by alterations in the membrane microenvironment of the receptors.

Increasing the incubation temperature greatly increased low-affinity binding in the presence of carbachol. When the effects of hexanol and increased temperature were combined, the increase in [^{3}H]QNB binding was much more limited at lower temperatures and markedly diminished at higher temperatures. There appeared to be an optimal degree of membrane fluidization near the body temperature without hexanol and at lower room temperature when membranes were further fluidized with hexanol. When fluidization with hexanol and with temperature were combined, the temperature for optimal binding was lowered to 31 °C. Higher degrees of membrane disorder, whether induced by hexanol or temperature, or both, caused a rapid decrease of binding below the baseline values. The homogenates from older mouse brains appeared to be more resistant to the disordering effect of hexanol, perhaps because their membranes were inherently more rigid.

The relationships between membrane structure, receptor binding, and aging could be conceptualized as a triangle, each factor affecting the other two. The effects of membrane fluidization with either alcohols or temperature on muscarinic receptor binding have been studied previously. Gurwitz and Sokolovsky (1980) used 50 °C for 5 minutes before assay incubation (25 °C) and found a 10- to 12-fold decrease in agonist, but not antagonist,

binding in mouse medulla-pons and cerebellum, but not in cortex. El-Fakahany and Richelson (1980) found that muscarinic receptor activation depended on temperature. Wei and Sulakhe (1982), who used a technique similar to the one described here, reported that cardiac muscarinic receptor binding at 37 °C, compared with 10 °C incubation temperatures, promoted conversion from high to low agonist affinity state receptor sites. However, heart and brain receptors may be different (Gibson *et al.*, 1983). Under appropriate conditions temperature and aliphatic alcohol's greater than ethanol increase the rate of binding to nicotinic receptors (El-Fakahany *et al.*, 1983).

Aging is associated with a decrease of shorter fatty acids, an increase of longer fatty acids, and a decrease in unsaturated fatty acids in brain phospholipids (Armbrecht *et al.*, 1983; Rouser and Yamamoto, 1969). This would tend to decrease membrane fluidity or increase viscosity. Direct measurements of microviscosity in mouse-brain membranes of several brain regions confirm that microviscosity increases as age advances (Samuel *et al.*, 1982). This increased membrane rigidity with advancing age also results in lesser disordering of membranes by ethanol in vitro (Armbrecht *et al.*, 1983).

The fact that the alcohols and their concentrations in these experiments are unphysiological is not considered important, because some physiological substances might alter the membrane order only in the restricted vicinity of the receptors in the same way that hexanol indiscriminately fluidizes all membranes everywhere. Physiologically relevant concentrations of ethanol in vitro do not change receptor binding. This probably means that changes in receptor binding are not related to acute intoxication. It does not mean, however, that prolonged in vivo exposure to ethanol could not change the membrane microenvironment of receptors and their function. All evidence taken together, aging appears to be associated with increased baseline membrane viscosity and with increased resistance to fluidization of membranes by alcohols. An increase in binding of low-affinity muscarinic receptors was induced by membrane fluidization with alcohols and with increasing incubation temperatures. This alcohol-induced increase in binding diminished with advancing age. We believe that this may be a link between age-related changes in membranes, receptors, and learning behavior.

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THE AGE-RELATED DECLINE IN ANTIBODY RESPONSE IS TRANSFERRED BY OLD TO YOUNG BONE MARROW TRANSPLANTATION

WILLIAM B. ERSHLER, DAVID L. ROBBINS, ANN L. MOORE, and JAMES C. HEBERT

Departments of Medicine and Surgery, Vermont Regional Cancer Center,
University of Vermont, Burlington, Vermont

Abstract—The immune response declines with age. This decline correlates with thymic involution and involves primarily a loss in T-cell function, whereas humoral immunity is more variably affected. In the current experiments we have measured immunoglobulin synthesis in vitro after mitogen stimulation, and specific antibody response after vaccination. We found that the response to pokeweed mitogen by non-specific immunoglobulin production, and the response to tetanus vaccine were deficient in old mice. With regard to the latter finding, the reduced response to vaccine was shown to be transferred to lethally irradiated young mice by old to young bone marrow transplantation. Both pokeweed mitogen and tetanus toxoid require T-cell help for optimal response, and, therefore, our observations are in accordance with the age-associated decline in T-cell immunity. The finding that young hosts transplanted with old bone marrow produce less antibody than young hosts transplanted with young bone marrow highlights the importance of the decline in cellular function with age.

Key Words: antibody response, bone marrow transplantation, cell function decline, immunoglobulin synthesis

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INTRODUCTION

THE IMMUNE deficiency that occurs with advancing age results primarily from age-associated changes in thymus-related (T-cell) functions (Makinodan and Kay, 1980). Age-associated changes in B-cell function have been reported (Callard and Basten, 1978; Segre and Segre, 1976) but are less prominent. It is probable, therefore, that reduced antibody production with age is most pronounced in those responses that require T-cell help (T-dependent) as suggested in early studies by Makinodan and Peterson (1962). We have been interested in the deficient antibody response observed in aged mice and humans after vaccine as it relates to reduced T-cell help (Ershler *et al.* 1984a; 1984b; 1985a). The primary involvement of *T* cells in the age-reduced antibody response was apparent in our studies of *T*-dependent systems such as tetanus toxoid (Willcox, 1974) and influenza (Virelizer, 1974) and *T*-independent systems such as pneumonococcal polysaccharides (Schiffman, 1983). The current studies were designed to further elucidate the reduced

Please address correspondence to Dr. Ershler, University of Wisconsin, 1300 University Avenue, Madison, WI 53706.
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antibody response with age, and to confirm that such changes were primarily the result of cellular and not environmental factors. The latter was accomplished by utilizing heterochronic, syngeneic bone marrow transplantation.

MATERIALS AND METHODS

Mice

Young (6 weeks to 3 months) C57 BL/6 mice were purchased from the Charles River Breeding Laboratories, Inc. (Wilmington, MA) and old mice were obtained through a pilot award from the National Institute on Aging (NIA) colonies bred at Charles River Breeding Laboratories and shipped to us at 22 months of age. The mice were certified pathogen free upon arrival, and while housed in our facility, they were maintained with regular photoperiods, isothermal conditions, and food and water *ad libitum*. At our facility the median survival for this strain (procured at 22 months), is approximately 28 months. Unless otherwise indicated, a minimum of eight animals was allocated to each group.

Immunoglobulin synthesis (in vitro)

Splenic cell suspensions from individual mice were prepared by gently processing splenic tissue in a Dounce homogenizer. One ml culture of 10^6 viable (by Trypan Blue exclusion) nucleated cells were cultured in RPMI 1640 containing 10% fetal calf serum, penicillin 100 μ/ml, streptomycin 100 μg/ml, and 1% pokeweed mitogen (PWM, GIBCO, Grand Isle, NY). After 6 days of culture at 37 °C in 5% CO_2, culture tubes were centrifuged and the supernatants were frozen for later determination of IgG and IgM level.

Enzyme-linked immunosorbent assay (ELISA) for IgG and IgM

IgG and IgM levels in the supernatants were assayed in an ELISA method adapted from Engvall and Perlmann (1971). Briefly, 96 well microtiter plates (Dynatech, Alexandria, VA) were coated with goat anti-mouse IgG or goat anti-mouse IgM (heavy chain specific, Tago Laboratories, Burlingame, CA) diluted 1:1000 in bicarbonate buffer (BB) (pH 9.6). The plates were incubated overnight at 4 °C and then briskly washed with phosphate buffered saline-tween 20(.05% v/V) (PBS-T). Two hundred μl of sample supernatants diluted 1:10 in PBS-T were added and the plates were incubated overnight at 4 °C. After a brisk wash with PBS-T, 200 μl of alkaline phosphatase conjugated goat anti-mouse IgG or IgM (Tago), diluted 1:2000 in PBS-T were added, and the plates once again were incubated overnight at 4 °C and, subsequently washed briskly with PBS-T. Two hundred μl alkaline phosphatase substrate (para-nitro-phenol phosphate, Sigma, St. Louis, MO) dissolved in diethanolamine buffer (pH 9.6) to a final concentration of 1 mg/ml, was added to each well. After 30 minutes incubation in room air enzymatic activity was measured spectrophotometrically (405 nm) utilizing a microELISA plate reader (Biotech, Burlington, VT). On each plate, standard serum samples with known quantities of IgG and IgM were added to construct a standard curve.

Vaccine protocol

Mice were injected with either tetanus toxoid (TT) (Wyeth, Marietta, PA) or a 14 valent pneumococcal polysaccharide vaccine (PN) (Pneumovax; Merck, Sharp and Dohme, West Point, PA). Stock TT was diluted 1:2.5 in sterile PBS and 0.1 ml was injected subcutaneously (sc). In certain experiments, a "booster" injection was given 4 weeks after the primary injection. This dose, schedule and route of injection has proven to be effective in producing a measureable antibody response (Ershler *et al.*, 1982). PN stock solution was diluted with PBS to a concentration of 2.5 μg/ml for each capsular type (including type 3) and administered intraperitoneally (ip). This concentration has been shown to be optimally immunogenic after ip injection (Baker *et al.*, 1971; Hebert *et al.*, 1985).

Antibody measurements

Heparinized blood samples obtained from the retroorbital plexus of each mouse before and every five days after immunization were separated by centrifugation, and the plasma was stored separately at −70 °C for later antibody determination. Antibodies specific for PN type 3 or TT were measured by an enzyme-linked immunosorbent assay as described (Ershler *et al.*, 1982; Hebert *et al.*, 1985). Briefly, for PN type 3, microELISA plates were coated with antibody prepared specifically to pn type 3 polysaccharide (anti-S. Pneumoniae serum, type 3; New York State Laboratories, Albany, NY). The antibody was diluted 1:50,000 in BB (300 μl per well), incubated overnight at 4 °C in a humidified chamber, and then washed briskly three times with a Water Pik (Teledyne) with PBS-T. Stock PN diluted 1:10,000 in PBS-T was added to each well (200 μl per well). Again plates were in-

cubated overnight at 4°C and washed with PBS-T, and each mouse plasma sample, diluted 1:1000 in PBS-T was added in quadruplicate wells (200 μl per well). After overnight incubation at 4°C and brisk washing with PBS-T, goat anti-mouse IgG and IgM alkaline phosphatase conjugate (TAGO) diluted 1:3000 in PBS-T was added to each well (200 μl per well) and incubated overnight at 4 °C. After a brisk wash with PBS-T, phosphatase substrate (Sigma) dissolved at 1 mg/ml in diethanolamine buffer (pH 9.6) was added to each well (200 μl per well). The reaction was stopped after 60 minutes with 50 μl of 3N NaOH and the color measured spectrophotometrically, as above.

A comparable ELISA for anti-TT was performed as described (Ershler *et al.*, 1982). MicroELISA plates were coated with TT diluted 1:1000 in BB. After overnight incubation at 4 °C, plates were washed with PBS-T, and mouse plasma diluted 1:1000 in PBS-T was added to quadruplicate wells (200 μl per well). The remainder of the assay was identical to that for anti-PN 3, as described above.

Heterochronic and homochronic bone marrow transplants

To test whether the observed reduced antibody response with age was the result of cellular, rather than environmental factors, antibody response was determined in heterochronic bone marrow transplant recipients. For this, young (2–3 months) mice were administered total body irradiation from a 1.2 MeV cobalt 60 (Theratron Junior; Atomic Energy of Canada, Ltd.) source. The mice were placed in a Lucite chamber and exposed at a dose rate of 43.8 rads per minute to a total dose of 950 rads. One hour later, these mice were administered bone marrow prepared from either young (2–3 months) or old (24–25 months) syngeneic healthy donors. To prepare the marrow the young or old donors were sacrificed by cervical dislocation and each femur was disarticulated and stripped of muscle. Small needle punctures were made at each end, and the marrow cavity was flushed with sterile 0.9% NaCl solution (saline). Bone marrow cells were washed in 0.1 *M* Dulbecco's PBS, pH 7.2, adjusted to 10^7 nucleated cells per ml, and injected (0.1 ml) into the lateral tail vein of recipient mice. Survival in the post-transplant period was generally between 50% and 75%, with the peak in death rate at 10 days, and no deaths occurring beyond 3 weeks post-transfusion. There was no difference in mortality between those given young bone marrow and those given old. In each experiment 3–5 mice were irradiated, but did not receive bone marrow transplant. None of these survived more than 10 days. By 3 weeks, surviving mice appeared healthy, and there was no weight difference between the experimental groups. At 2 months post transplant, mice were vaccinated with either TT, PN, or both. We had previously shown that the specific antibody response in animals receiving both vaccines was the same for each antigen when compared to the antibody response in animals receiving just a single vaccine (Ershler *et al.*, 1985a).

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Statistical analysis

Antibody levels for each mouse at each interval after immunization were standardized against reference sera and averaged, and the difference between groups was assessed by the Student's *t* test. Significance was determined at a probability value less than 0.05.

RESULTS

In vitro Immunoglobulin synthesis

Splenic lymphocytes from 24 mice of various ages were cultured in the presence of PWM (a T-dependent *B*-cell mitogen) for 6 days. The immunoglobulin synthesized and secreted into the culture supernatant was measured by an ELISA as described above. Although the numbers of mice in each group were small, there appeared to be less IgG and IgM present in the cultures from the very young and old mice (Figure 1).

Antibody response to vaccine

The importance of the above observation would become apparent if it could be shown that the reduced nonspecific immunoglobulin produced in response to mitogen actually reflects reduced specific antibody response to antigen. To test this, we vaccinated three groups of mice (5, 16, and 23 months of age; n = 6 in each group) with tetanus toxoid. Specific anti-tetanus antibody was measured by an ELISA, as described above, and the results are depicted in Figure 2. In this experiment the plasma from the mice in each group

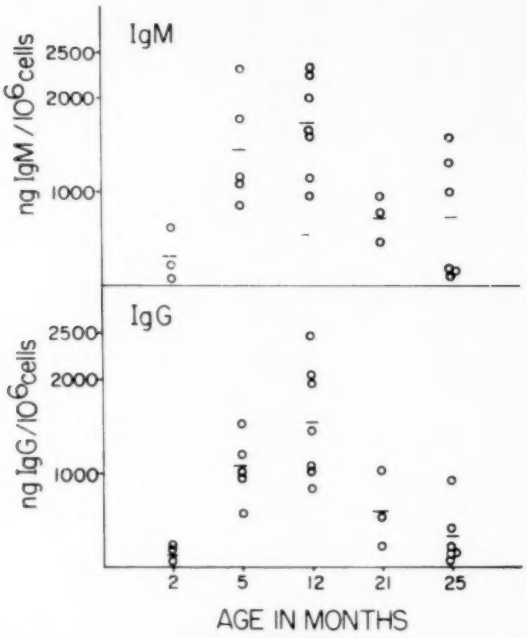

FIG. 1. Immunoglobulin synthesis in vitro. Splenocytes from mice of different ages were cultured for six days in the presence of PWM. IgG and IgM synthesized and secreted into the culture supernatant were measured by ELISA. Lower levels of IgG and IgM were observed in the very young and old mice. (Horizontal bars represent the mean value for the data shown.)

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was pooled, and the antibody content pre- and post-vaccine determined. The value depicted in Figure 2, therefore, is the actual measured level for each pooled sample. It is apparent that the greatest increment in antibody level post vaccine was in the young mice, and the smallest increment (poorest response) was in the old mice. In subsequent experiments, antibody level was determined individually in all mice, and statistical comparisons between the different groups were then possible.

Antibody response in hetero- and homochronic transplant recipients

Young mice received lethal irradiation and were reconstituted syngeneically with either young or old marrow. The rate of successful engraftment (survival) was equal in both groups, approximately 70%. Two months after transplant mice were vaccinated with TT. Plasma was sampled for antibody content prior to, and every two weeks thereafter for six weeks. A second "booster" injection of TT was administered one month after the first. The antibody response of these groups was compared to that of control, and it is depicted in Figure 3. As can be seen, young mice reconstituted with young marrow had an antibody response very similar to age matched controls that had not received radiation or bone marrow transplant. Those young mice that had received old marrow, however, had a less prominent primary and secondary response.

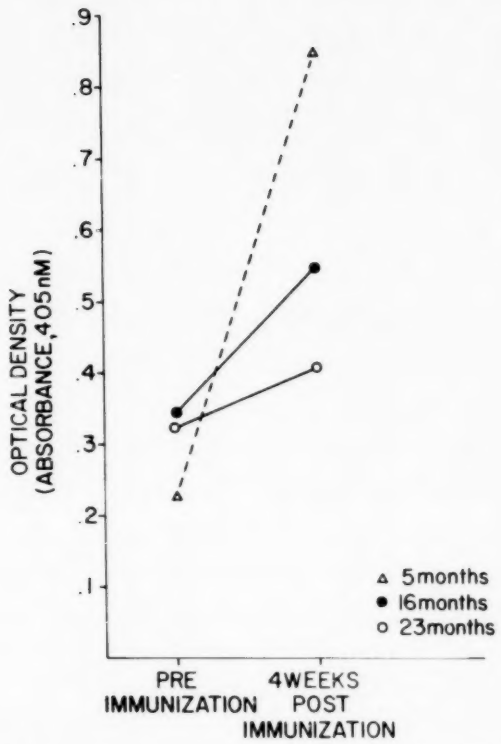

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FIG. 2. Antibody response to primary tetanus toxoid immunization. There were six mice in each age group and the plasma from these mice was pooled. The antibody content in each pooled plasma sample was measured by ELISA. It was apparent that the difference between pre- and post-immunization antibody levels was greatest for the young mice and least for the old. In subsequent experiments antibody levels from individual mice were determined, and statistical analyses employed.

In a repeat experiment, transplant recipients were vaccinated with both TT and PN. In earlier studies in our laboratory, we had demonstrated that concurrent vaccination did not alter specific antibody response to either antigen (Ershler *et al.*, 1985). The results with reference to anti-tetanus antibody (Figure 4) are similar to those from the first experiment. The change in antibody level pre- and post-vaccine (as indicated by the area between pre- and post-vaccine antibody dilution curves (Murphy *et al.*, 1981)) was significantly less ($p < 0.05$) for those mice reconstituted with old marrow, when compared to those reconstituted with young. The antipneumococcal antibody results, however, were quite different (Figure 5). In this T-independent system, no significant difference in antibody response among the different groups was observed.

DISCUSSION

In these experiments we found that in vitro immunoglobulin synthesis after a nonspecific stimulus (PWM) was decreased in old mice. We also found that the specific anti-

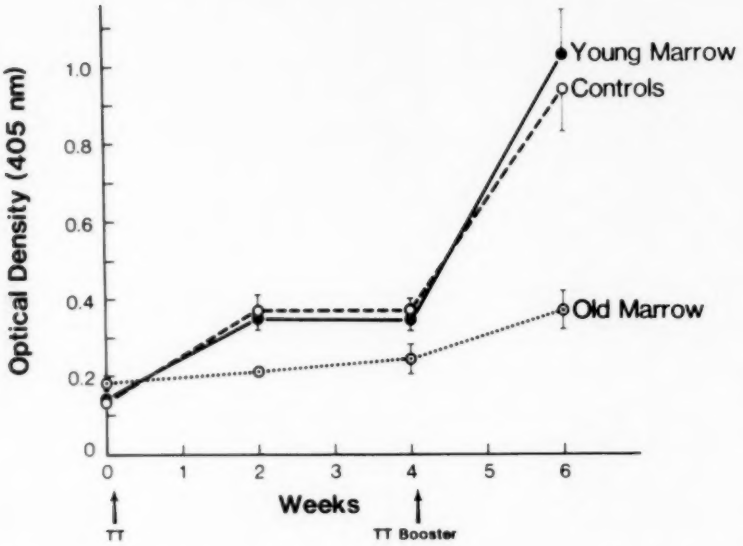

FIG. 3. Anti-TT antibody levels in hetero- and homochronic bone marrow transplant recipients. Anti-TT antibody levels from each individual mouse at each time point was determined, and the mean (+/−SEM) is depicted (n = 10 in control group and 9 in each transplant group). Young mice reconstituted with young bone marrow had greater primary and secondary antibody responses to TT when compared to those young mice reconstituted with old marrow. ($p < 0.05$ at each post-vaccination time point.)

body response to vaccination (TT) was reduced in old mice. These findings correlate with what has been described by other investigators for other antigen systems in mice (Segre and Segre, 1976; Callard and Basten, 1978; and Makinodan and Kay, 1980) and by us for TT and influenza in humans (Ershler *et al.*, 1984a, 1984b).

The observed reduced specific antibody response could result from a number of host factors including those involved in antigen processing and presentation as well as other non-immune, microenvironmental factors. The information gained from the bone marrow transplantation experiments, however, confirms the importance of cellular senescence. Young mice reconstituted with old marrow produced significantly less anti-tetanus antibody than did young mice reconstituted with young marrow. The failure to demonstrate this with pneumococcal polysaccharide antigens (PN) suggests that, with T-independent antigens (such as PN), less of an age-effect is observed. This supports the contention that it is a senescence of T-cell function that accounts for the observed reduced antibody response to TT. Nevertheless, a specific T-cell defect has not been clearly defined. Despite the age-preservation of hematopoietic progenitor cells (Harrison, 1983), T-cell progenitors (in mice) do decline with age (Tyan, 1977). Furthermore, utilizing heterochronic parabiosis, Kay (1984) was able to demonstrate an inability of progenitor cells from old mice to migrate and mature through young thymic tissue, suggesting that an extra-thymic regulatory mechanism may account for some of the age-related changes. Nevertheless, several functional T-cell defects have been described (for review see Makinodan and Kay, 1980), including reduced Interleukin-2 (IL-2) production (Gillis *et al.*, 1981; Ershler *et al.*, 1985b). This latter observation may reflect a reduction in IL-2

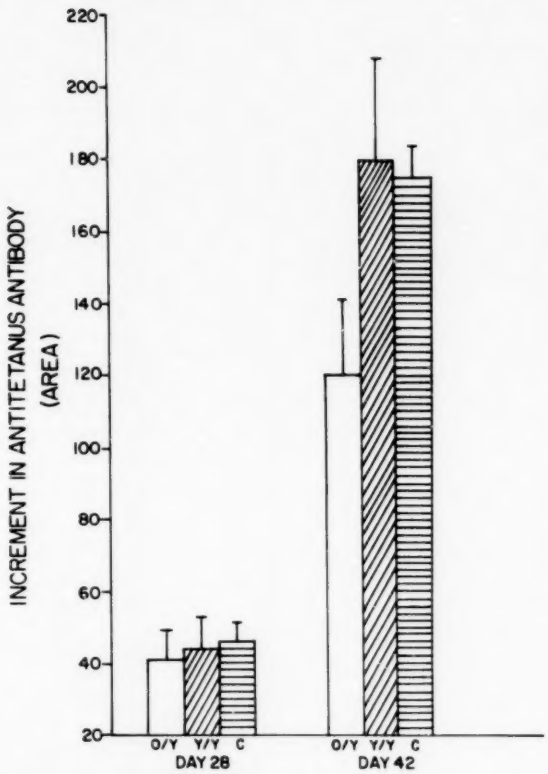

FIG. 4. Anti-TT antibody response in bone marrow transplant recipients transplant recipients. Data from the repeat experiment is depicted. Antibody response is defined as the calculated area between pre- and post-immunization dilution curves (ELISA) as described by Murphy *et al.* (1981). Area is determined for each mouse and the mean (+/−SEM) for each age group is depicted. (O/Y, young mouse with old bone marrow, $n = 9$; Y/Y, young mouse with young bone marrow, $n = 10$; C, young non-irradiated, non-transplanted control mice, $n = 10$.) In this experiment there was no difference at day 28 (unlike the previous experiment). Two weeks after booster injection, however, recipients of young marrow had significantly ($p < 0.05$) greater response than recipients of old marrow.

producing cells rather than a decline in IL-2 production per cell (Miller and Stutman, 1982).

It is therefore probable that the observed deficient antibody response is attributable, to a large extent, to a defect (either quantitative or qualitative) in T-cell help, and not to B-cell, macrophage, or microenvironmental factors. In this way our findings are conceptually consistent with the age-associated "intrinsic defect" explanation for the observed reduced immune function (Price and Makinodan, 1972; Averill and Wolf, 1985). Nevertheless, the observations do not contradict the hypothesis of Astle and Harrison (1984) that suppressor factors, operational in old bone marrow, inhibit immune response.

If similar mechanisms account for reduced antibody response in people, it may be of clinical importance. It is considered dogma that antibody response is an important host protective mechanism, and accordingly, vaccination programs have been vigorously en-

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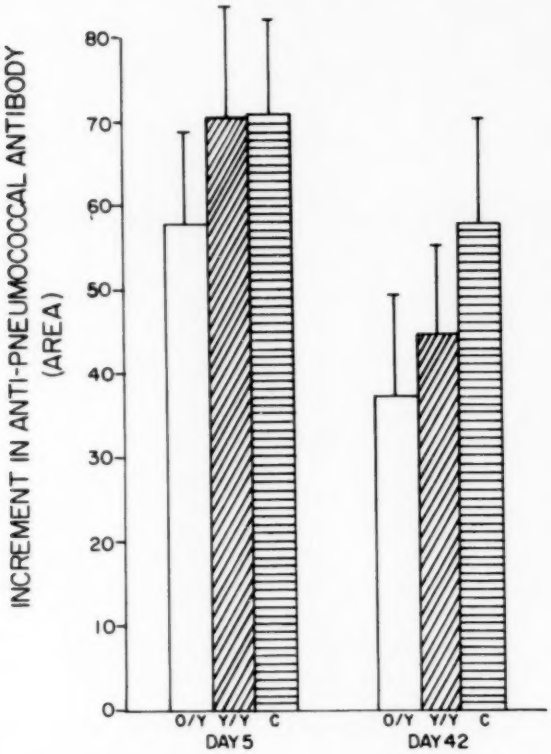

FIG. 5. Anti-pneumococcal polysaccharide (PN) antibody response in bone marrow transplant recipients. Experimental groups were as discussed above. Unlike the anti-TT response, no significant differences in antibody response to PN were observed.

dorsed in most countries. In fact, such programs may be the most successful of all preventive health measures. Nevertheless, in the elderly, in whom certain vaccinations are frequently administered, the response is often times deficient, and protection is not afforded (D'Alessio *et al.*, 1969). For example we observed that the response to influenza vaccine in 1/3 of a sample of fully ambulatory, "well" people over the age of 70 years was deficient (Ershler *et al.*, 1984b) and similar findings have been observed in other laboratories (M.E. Weksler, personal communication, 1985). Elucidating the mechanism for this reduced response would be of importance for the future design of immuno-restorative measures.

Acknowledgments—This paper was supported by grants from the National Institute of Health (CA22435, AG00332) and The American Federation for Aging Research.

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PUBLICATIONS OF INTEREST

B.B. BOGOMOLETS

FRONTIERS IN MEDICINE: IMPLICATIONS FOR THE FUTURE

R.J. Morin and R.J. Bing, Editors. New York: Human Sciences Press, Inc., 1985, 332 pages. ISBN 0-89885-209-9.

Despite the title, this book is intended for an audience of biogerontologists and those interested in the chronic diseases that afflict the elderly. The chapters are authored by attendees at a conference sponsored by the American Longevity Association which is "dedicated to sponsoring creative research and educational programs in aging and the age-related disorders of heart disease, cancer and diabetes."

The 20 chapters are divided into three groups: The Major Diseases, General Medical Advances, and The Aging Process. The latter is covered by L. Hayflick (Cell Biology), C.E. Finch (Neurobiology), T. Makinodan (Immunology), N.W. Shock (Physiology), and R.N. Butler (The Science and Art of Living Long and Well).

Atherosclerosis, cardiovascular diseases, cancer, and diabetes are represented by such luminaries as R.W. Wissler, R.J. Morin, D. Kritchevsky, D.H. Blankenhorn, R.J. Bing, T.L. Vander Laan, M.E. DeBakey, R. Jarvik, M.B. Shimkin, B.S. Blumberg, W.T. London, A.V. Schally, T.W. Redding, A.M. Comaru-Schally, and E.R. Arquilla.

General medical advances yield to the scrutiny of U.S. von Euler, R.A. Good, N.K. Day, and R.J. Glassock.

A good overview by folks who should know.

BEYOND THE HELIX: DNA AND THE QUEST FOR LONGEVITY

Carol Kahn. New York: Times Books, 1985, 286 pages. ISBN 0-8129-1153-9, $17.95.

Dust jackets of books intended for the mass market are seldom known to be examples of objectivity, modesty, or accuracy. This one is no exception.

"*Beyond the Helix*" tells the story of the trailblazing scientists who have taken up the challenge of unlocking man's greatest mysteries—why we age and whether we can retard the aging process and extend life . . . Carol Kahn gives us an illuminating portrait of the people working at molecular biology's frontier as well as a rare behind-the-scenes examination of how scientific discoveries are made. The result is the first lay account of some of

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the most revolutionary research being conducted in modern science." Actually, there have been several lay accounts of modern research in biogerontology, the most recent of which is *Prolongevity II* by A. Rosenfeld. And it is a far more objective and authoritative book. [See review in *Experimental Gerontology* **20,** 308 (1985)]

What will come as a surprise to most biogerontologists who will read this book is that Dr. Ronald Hart emerges as the figure who has made the greatest recent contributions to the field and around whom everyone else and their work seems to revolve.

It is difficult to understand how this extraordinary view evolved but it is not difficult to understand how, once made, it has taken on a life of its own in the hands of a competent writer. And Carol Kahn is a competent writer. She has a frisky, chatty style even when explaining some subtle nuances in molecular biology. The action flows smoothly but bristles, unfortunately, with the kind of manufactured excitement that lay authors of books and screen plays insist must occur in research laboratories. When is the last time that you ran down the halls shouting "Eureka!"? Never? Then, you must have never experienced the thrill of discovery. Perhaps just the agony of defeat? Not so, many of Carol Kahn's characters.

Although this book is not intended to inform practicing biogerontologists of new advances in their field, it was on sale at a separate booth during the last annual meeting of the Gerontological Society of America. (Yes, I received a passport to attend.) Perhaps its appeal to the cognicenti might be its enormous content of gossip in the genre made famous by Jim Watson in "*The Double Helix.*" I learned more about who dislikes whom, and why, than I have in all those hours spent nursing glasses of vodka with colleagues who work in this field. There is enough gossip here for a "Dallas" TV mini-series.

The factual blunders in this book, that I know are spurious, are sufficiently numerous to make me worry about the accuracy of much that is written. For example, we are introduced to "The protein error theories of Leslie Orgel and Roy Medvedev. . . ." Roy Medvedev, a Russian historian, is the twin brother of Zhores Medvedev, the gerontologist whose name was undoubtedly intended. (I know this to be true because I have seen Roy recently in Moscow.)

Kahn claims that the out of court settlement that Hayflick won in his suit against the NIH yielded $900,000. This suit, filed before the Chakrabarty decision, established the precedent that biologists supported with federal funds have property rights to materials they might invent, like novel cells or microorganisms. Had the suit not been won, establishment of the thriving biotechnology industry would have been seriously compromised because they use many cell populations and microorganisms originally developed with federal funds at academic institutions. Kahn makes an order of magnitude error. The actual sum was $90,000 and all of that, plus more, went for legal expenses.

If, for some reason, you are a Ron Hart groupie you will adore this book. If not, you will have to wait for that elusive definitive and objective book to be written. Meanwhile, read *Prolongevity II.*

THRESHOLDS IN AGING: THE 1984 SANDOZ LECTURES IN GERONTOLOGY

M. Bergener, M. Ermini, and H.B. Stahelin, Editors. Orlando, FL: Academic Press, Inc., 1985, 301 pages. ISBN 0-12-090160-9, $25.00.

Through the generosity of Sandoz Ltd, Basel, Switzerland, the "Sandoz Prize for Gerontological Research" has been established. The prize of 20,000 Swiss Francs is awarded by

a jury composed of the Executive Committee of the International Association of Gerontology. The award is made biannually to encourage "research in all areas of gerontology and geriatric medicine including biological, medical, psychological, social and other relevant aspects with special emphasis on multidisciplinary research programs."

This book contains chapters written by the two winners, whose groups shared the first prize in 1984, plus the runners-up. In consequence of the broad area of gerontology and geriatrics that this award embraces, the contributions cover such diverse fields as genetics, post-transcriptional events, immunology, animal models, cell aging, neurobiology, Alzheimer's disease, pharmacology, longitudinal studies, psychometrics, and cognition. The book is a smorgasbord. Like all proper smorgasbords it is unfocused but has at least something that will appeal to all tastes. You will probably go home hungry unless you prefer to dine only on appetizers. There is no main course.

DRUGS AND AGING

D. Platt, Editor. Berlin: Springer-Verlag, 1986, 216 pages. ISBN 3-540-15913-4 and 0-387-15913-4.

This collection of articles is written by participants at the 2nd International Erlangen-Nurnberg Symposium on Experimental Gerontology, 28 November to 1 December, 1984. The contributions do not, as the title may suggest, cover the use of illicit drugs by the elderly. Rather, the contributors write on prescription drug actions and interactions at the molecular, cellular, organ, and organism levels.

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The editor quite rightly says, "By contrast with our extensive knowledge of pharmacokinetics and pharmacodynamics in younger age groups, few facts are available in respect of the elderly." This observation becomes even more important when it is realized that the elderly consume more drugs, proportionately, than does any other age group and that drug trials for safety and efficacy have historically been conducted only in healthy young adults. It is inevitable that this must change.

Twenty chapters compose this book. The first is by Z.A. Medvedev who provides us with a characteristically thoughtful piece on "Age-Related Changes of Transcription and RNA Processing." L. Hayflick follows with a primer on cell culture and how in vitro drug testing might be enhanced. A. Macieira-Coelho and B. Azzarone discuss "Aging, Cancer, and the Life Span of Cells" in less than ten pages. Subsequent articles cover aspects of aging on drug metabolism, liver detoxification, and the pharmacodynamics and pharmacokinetics of selected drugs used by the elderly.

This is a good overview of a field that will command even greater attention as the magnitude of the problem becomes better appreciated. Polypharmacy may very well become the next major target of interest in gerontology. It may replace Alzheimer's disease as the next growth stock. Remember, you read it first here.

BEHAVIOR AND PATHOLOGY OF AGING IN RHESUS MONKEYS

R.T. Davis and C.W. Leathers, Editors. New York: Alan R. Liss, Inc., 1985, 380 pages. ISBN 0-8451-3407-8, LC 85-5251, $88.00.

This is volume 8 in a series entitled *Monographs in Primatology*. Eighteen chapters are divided under headings that include "History of Project, Basic Pathology, and Cell

Growth," "Brain, Sense Organs and Behavior," "Endocrine System," "Heart and Lung," and "Bone and Muscle."

The observations reported are "based on a 29 year study of rhesus monkeys." The program, it is said, is unique because there exists "no such long-term chronicle of human aging in the scientific literature . . ." and "the animals used closely reflect purely age related changes in behavior and pathology" because of ". . . a series of strict controls (i.e., familiarity with the animals' histories, freedom from trauma, proper diet and exercise, lack of physiological interventions and daily problem solving). . . ."

Although the Baltimore Longitudinal Study of Aging in humans is only a mere 28 years old, and no strict control of life style is possible, the participants histories are well known. Freedom from trauma or physiological interventions, however they may be defined, is easily controlled by the proper recording of patients' histories and eliminating them from the data set, if that is truely desireable. "Proper diet and exercise" for humans and monkeys, as it relates to longevity or maintenance of optimum health, is substantially unknown. Is "daily problem solving" by the monkeys somehow controlled as inferred? If so, why? What evidence is there that qualitative or quantitative differences in daily problem solving effects longevity?

In any case, the studies reported here are useful and informative. The length and variety of the studies may be unique for monkeys but certainly not for humans as is stated.

The monkeys studied here, like the rodents used in aging research, are so well controlled in captivity that they actually age—something that does not occur in their natural environment. Is an artifact only associated with captivity being studied?

EXTRACELLULAR MATRIX: STRUCTURE AND FUNCTION

A. Hari Reddi, Editor. New York: Alan R. Liss, Inc., 1985, 458 pages. ISBN 0-8451-2624-5, LC 85-5264, $96.00.

These 38 chapters are contributed as the "Proceedings of a Dow-UCLA Symposium" held in Keystone, Colorado, April 22–29, 1984. Coverage includes Collagens: Structure and Biosynthesis, Elastin, Proteoglycans, Fibronectin and Laminin, Extracellular Matrix and Development and Extracellular Matrix and Diseases. This is volume 25 in the UCLA Symposia on Molecular and Cellular Biology, New Series. The publishers' puff piece states "that a comprehensive understanding of cell biology must incorporate studies of the extracellular matrix . . ." and this volume does cover aspects of extracellular matrix chemistry as it applies to the structure and function of animal cells. The animal cells studied are those associated with embryology, development and various disease states such as cancer, diabetes, and wound healing. No mention is made in the 458 pages of the role of the extracellular matrix in aging. So much for the claim of "comprehensiveness."

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ANNOUNCEMENT

Caenorhabditis Genetics Center

GENETIC STOCKS of *Caenorhabditis elegans* are available for use by investigators initiating or continuing research on this nematode. The Center's collection of more than 1000 strains includes at least one allele of each identified gene, all available chromosome rearrangements and selected multiple mutant stocks useful for genetic mapping.

The functions of the Center include coordination of genetic nomenclature, maintenance and distribution of a bibliography of research publications, and annual revision and distribution of the *C. elegans* genetic map (see Swanson, M.M.; Edgley, M.L.; Riddle, D.L. (1984). *Caenorhabditis elegans* in *Genetic Maps 3*, O'Brien, S.J., ed. New York: Cold Spring Harbor Press, pp. 286–299). The Center acts as the central clearinghouse for naming strains, alleles, and genes. A computerized database stores the bibliographic information and the data used to generate the genetic map, as well as descriptive data on strains. The Center also distributes a *C. elegans* research newsletter by subscription.

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The goal of the Center is to promote accumulation and documentation of the genetic wealth of *C. elegans* for all types of biomedical research on this organism. Established in 1979 under sponsorship of the National Institute on Aging, it is the only central repository for *C. elegans* strains. Requests for strains should include a brief statement of the research and/or research training activity for which the stocks are intended. Recipients affiliated with non-profit organizations are asked to pay postage costs, to subsequently acknowledge the Center in publications resulting from use of the strains, and to provide the Center with reprints of those publications. Inquiries should be addressed to:

M.L. Edgley, Curator or
D.L. Riddle, Director
Caenorhabditis Genetics Center
Division of Biological Sciences
Tucker Hall
University of Missouri
Columbia, Missouri 65211

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ANNOUNCEMENT

The Third Tokyo Symposium on Liver and Aging

THE THIRD Tokyo Symposium, *Liver and Aging*, will be held in Tokyo, Japan on August 20 through 22, 1986 with additional coverage of *Liver and Brain*. The primary objective of the symposium is to discuss the interactions and functional alterations of these two organs as they are affected by aging. The symposium will be limited to about 100 participants. The total number of speakers will be less than 30.

Though participation will generally be limited to predetermined invitees, a small number of interested and qualified guests will be admitted both as audience and speakers. Financial support will be provided for all invited speakers from outside Japan. Those who wish to present papers are encouraged to submit an abstract to the organizer. Papers most appropriate to the subject will be given priority.

The majority of the presentations will be on the pharmacodynamics of drugs affecting the central nervous system, including drug metabolism, pharmacokinetics, end organ sensitivity changes during aging, and neural and humoral control of the aging liver. Papers covering the aging of only a single organ (liver or brain) may be included but the number of such papers will be limited.

Those interested in the symposium are kindly requested to contact the organizer.

THIRD TOKYO SYMPOSIUM *LIVER AND AGING* and *LIVER AND BRAIN*

Place: Hotel Pacific, Takanawa, Tokyo, JAPAN

Date: August 20–22, 1986

Abstract Deadline: February 28, 1986

Organizer: K. Kitani, M.D.
First Laboratory of Clinical Physiology
Tokyo Metropolitan Institute of Gerontology
35-2, Sakaecho, Itabashi-ku
Tokyo, JAPAN (Telephone 03-964-1131, Ext. 3091)

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ANNOUNCEMENT

The International Association of Gerontology Announces the Third Sandoz Prize for Gerontological Research

THIS PRIZE, worth 20,000 Swiss Francs, is sponsored by SANDOZ LTD, Basel, Switzerland. Its purpose is to encourage research in all areas of gerontology and geriatric medicine including biological, medical, psychological, social, and other relevant aspects with special emphasis on multidisciplinary research programs.

The Jury consists of the Executive Committee of the International Association of Gerontology, its official Sandoz Prize coordinator, and one representative of Sandoz Ltd. The prize will be awarded on the occasion of the 1st European Congress of the International Association of Gerontology to be held in Brighton, United Kingdom, September 1987.

Applications in English should comprise a summary of the research work in 3–5 pages. Include a curriculum vitae, bibliography, and reprints of not more than three pivotal publications in English, or with extended summaries in English.

Individuals, research teams, or institutions are invited to submit their applications not later than 31 October 1986 to Prof. M. Bergener, M.D., International Association of Gerontology, Rheinische Landesklinik, Wilhelm-Griesinger-Strasse 23, D-5000 Köln 91, Federal Republic of Germany.

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OBITUARY

Sir Frank MacFarlane Burnet
1899–1985

Sir Frank MacFarlane Burnet, who was an Associate Editor of *Experimental Gerontology*, died peacefully on August 31, 1985. He was a joint recipient of the 1960 Nobel Prize for Physiology and Medicine and was internationally recognized as the originator of the clonal selection theory of immune response, which forms the basis of modern immunology.

Burnet began his career in human biology shortly after completing his medical training in 1922 by becoming involved in the etiology of infectious disease. Early in his career he demonstrated his interest in general biological principles by writing a theoretical paper on the ecology of host-parasite relationship with a focus on viral diseases (Burnet, 1936). As an experimentalist, his efforts in improving the techniques for growing viruses in chick embryos led to the establishment of a quantitative method for studying viral replication (Burnet, 1934) and to the identification of a rickettsia-like organism as the causative agent of Q fever (Burnet and Fenner, 1937). The organism was therefore named *Rickettsia burnetii* and changed subsequently to *Coxiella burnetii*.

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Burnet's interest in immunology was a natural outgrowth of his commitment to understand viral diseases, and it was his unique Darwinian approach to the cellular processes within the body which led, in the mid 50's, to his concept of somatic mutation and selective survival among somatic cells of the body. In 1957 he published two major papers: one was on clonal selection theory of immunity (Burnet, 1957a) and the other on the biology of cancer (Burnet, 1957b). Needless to say, as with many other aspiring immunologists, I was influenced very much by his thought-provoking clonal selection theory of immunity.

The elaboration of the clonal selection theory, its extension to autoimmune disease and the idea of immune surveillance against incipient cancer logically led him into gerontology, which had been his chief interest since 1969. His major contribution to gerontology was his theory on the role intrinsic mutagenesis plays in aging, which he formulated in a series of lectures in the early 70's and published in a text in 1974 (Burnet, 1974). His last publication on aging was perhaps the chapter on "Age-Associated Heredo-Degenerative Conditions of the Central Nervous System" which he wrote in 1983 (Burnet, 1983), and its focus was on the role intrinsic mutagenesis plays in diseases of the central nervous system.

In developing his intrinsic somatic mutation theory on aging, Burnet clearly demonstrated his creative ability to synthesize reductionistic and demographic data into a general biological principle. This can be seen in his book on *Intrinsic Mutagenesis, A Genetic Approach to Ageing* (Burnet, 1974), which should be mandatory reading for all aspiring gerontologists. It would appear that Burnet was influenced heavily by: (a) Monod's book on *Chance and Necessity* (1972), which he considered to be the modern restatement of Darwin's evolutionary philosophy; (b) Burch's stochastic mathematical approach to the

demography of age-related degenerative disease (1968); (c) the demographic data on cancer demonstrating that the distribution with age has a stochastic regularity, irrespective of the intensity of environmental factors, which points to an intrinsic and random process (Burnet, 1974); and (d) the phenomenon of the Hayflick limit demonstrated *in vitro* by Hayflick (1965) and *in vivo* by Williams and Askonas (1972). In brief, his hypothesis on aging can be stated in three propositions:

1. Average lifespan is determined by the rate at which somatic mutations accumulate in the body.
2. Mutation results from error in the replication or repair of DNA.
3. Degree of error-proneness depends on the structure of the enzymes concerned in repair and replication of DNA.

Sophisticated techniques of molecular genetics are now available to experimentally examine and test his theory of aging. Regardless of the outcome of the experimental results, Burnet will always be recognized as a pioneer in applying Darwinian principles to the understanding of cellular aging.

Takashi Makinodan
Associate Editor
Director, Geriatric Research, Education and Clinical Center
VAMC West Los Angeles
Professor of Medicine in Residence, UCLA
Adjunct Professor of Biology, USC

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AGING MODIFIES THE EXPRESSION OF HEPATIC MICROSOMAL CYTOCHROMES P-450 AFTER PRETREATMENT OF RATS WITH β-NAPHTHOFLAVONE OR PHENOBARBITAL

JUNQUI SUN,[1] PAUL P. LAU, and HENRY W. STROBEL[2]

Department of Biochemistry and Molecular Biology,
The University of Texas Medical School at Houston, Houston, Texas 77225

Abstract—The various forms of hepatic cytochrome P-450 respond differentially to aging and induction. We examined the levels of six forms of cytochrome P-450, designated as Forms 1 through 5 and Form b, as a function of age and induction. Radial immunodiffusion analysis of rat liver microsomes indicate that cytochrome P-450 Forms 1 and 2 respond to induction by β-naphthoflavone or phenobarbital less well in aging rats than in young rats. β-naphthoflavone is less effective in inducing Forms 3, 4, and 5 in aging rats than in young rats. Phenobarbital, however, is more effective in inducing Forms 3 and 4 in aging rats than in young rats but does not induce Form 5 in either young or aging rats. Although Form b is induced predominately by phenobarbital, β-naphthoflavone induces Form b moderately in aging rats. Phenobarbital induces Form b to approximately the same extent in aging rats and in young rats. In untreated rats Form 2 is the predominant form, while Forms 1 and 3 are present in moderate amounts. The results of the immunodiffusion analysis were confirmed by the resolution and partial purification of cytochromes P-450 from microsomes of aging and young rats pretreated with β-naphthoflavone or phenobarbital. These results identify changes with age in specific forms of cytochrome P-450 as a function of the aging process in rats.

Key Words: cytochrome P-450, drug metabolism, liver aging, hepatic microsomes

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INTRODUCTION

MANY CLINICAL reports have described the progressive loss with age of drug metabolizing capacity as reflected in increased sensitivity to drug treatment dosage levels and in a concomitant increased sensitivity to toxic side effects of drugs (Schmucker, 1979; Gillette, 1979). These observations have been confirmed and studied further in experimental animal model systems by a number of workers (Kato and Tanaka, 1968; Rikans and Notley, 1982a). Kato and Tanaka (1968) demonstrated that phenobarbital induced higher

[1]Present address: Department of Pharmacology, Beijing Second Medical School, Beijing, People's Republic of China.

[2]Author to whom correspondence should be addressed; recipient of Research Career Development Award KO4 CA00311.

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levels of drug metabolism activity in young rats than in older rats. Drug metabolism, defined as the activation or inactivation of various xenobiotics resulting in an increase or decrease, respectively, in pharmacological effects, is primarily a function of the liver and specifically the endoplasmic reticulum or microsomal subfraction (Brodie *et al.*, 1958). The cytochromes P-450, a family of related proteins, function as the catalysts in the activation/inactivation reactions and confer substrate specificity to the reaction. Rikans and Notley (1982a) confirmed the decrease in phenobarbital-inducible benzphetamine demethylation activity with age, but pointed out that β-naphthoflavone-inducible *p*-nitroanisole activity was higher in older rats than in young rats. Thus, effects of aging on liver activities seem related to substrate and induction parameters. Since a principal response to inducers is the appearance of, or increase in cytochrome P-450 and since substrate specificity is conferred by cytochrome P-450, it is likely that these differential effects of aging are drug mediated or modulated through effects on cytochrome P-450.

Sodium dodecyl sulfate gel electrophoresis studies by Rikans and Notley (1982a, 1982b) of hepatic microsomes from young and old rats treated with phenobarbital or β-naphthoflavone suggest that certain bands in the cytochrome P-450 region increase or decrease with age. A comparison of age and induction on the amount of two forms of cytochrome P-450 in rabbit liver microsomes has been reported by Chiang *et al.* (1982). These workers show, using immunoquantitation techniques, that two isozymes of rabbit liver $P\text{-}450_{LM2}$ and $P\text{-}450_{LM4}$ decline with age.

We have recently shown that unlike the liver, extrahepatic cytochrome P-450 mediated drug metabolism system activities remain more constant and show the same or increased inducibility with advancing age (Sun and Strobel, manuscript submitted). Further, benzo[α]pyrene hydroxylation in liver and the extrahepatic tissues was correlated with age and with changes in the amounts of cytochrome P-450 Form 5 (Form c). Form 5 is one of five forms isolated from hepatic microsomes of β-naphthoflavone pretreated rats (Lau and Strobel, 1982) and has the highest reported turnover for benzo[α]pyrene.

In this present report, we document the changes in cytochrome P-450 composition of rat liver with age by identifying, separating, and partially purifying six forms of cytochrome P-450 in hepatic microsomes from rats of various ages after pretreatment with phenobarbital and β-naphthoflavone in order to relate changes in metabolic activity to specific changes in cytochrome P-450 composition.

MATERIALS AND METHODS

Preparation of Microsomes

Hepatic microsomal fractions from male Sprague-Dawley rats of different age groups were prepared as previously described (Saito and Strobel, 1981; Gum and Strobel, 1979). The rats were obtained shortly before use from the colony of aging Sprague-Dawley rats which were maintained for The National Institute on Aging by Charles River Laboratories. The young (2–4 week old) rats and the aging (one year old) rats were treated with β-naphthoflavone or phenobarbital before sacrifice as described (Haugen and Coon, 1976; Ryan *et al.*, 1975). These two age groups were selected because they represent very different segments of the aging spectrum.

Separation and purification of the multiple forms of cytochrome P-450

The scheme employed for resolution and purification of the multiple forms of cytochrome P-450 is essentially the same as that described earlier by our laboratory (Lau and

Strobel, 1982). In general, the Renex-690 solubilized microsomal fraction was applied to a Sephadex A-25 column to separate the cytochrome P-450 fraction from the cytochrome P-450 reductase fraction. The cytochrome P-450 fractions contained some cytochrome b_5 which was further removed by the subsequent DE-53 column. The resolution of the multiple forms was performed with a DE-53 column at room temperature as described (Saito and Strobel, 1981; Lau and Strobel, 1982).

Sodium dodecyl sulfate electrophoresis was performed as described by Laemmli *et al.* (1970). Cytochrome P-450 was quantified using the CO-difference spectrum described by Omura and Sato (1964).

Immunodiffusion, either radial or Ouchterlony, was performed as described by Thomas *et al.* (1979). The results obtained from immunoradial quantitation were in agreement with those obtained by immunoelectrophoresis ("Rockets") as we have shown earlier (Strobel and Lau, 1982) with the procedure described by Pickett *et al.* (1981). In all the immunoquantitation methods, gel-bound films (FMC) were used as described (Strobel and Lau, 1982; Lau *et al.* 1982). Antibodies were prepared as described by Strobel and Lau (1982) using purified forms prepared from 10-week-old rats. Protein concentrations were determined by the method of Lowry *et al.* (1951). All chemicals used were reagent grade or better.

RESULTS

In order to define the age-related changes in cytochrome P-450 content and composition, we have used several qualitative and quantitative approaches. Estimation of the amounts of various forms of cytochrome P-450 present as a function of age was accomplished by a radial immunodiffusion technique using antibodies to purified hepatic cytochromes P-450. In order to test for the presence of age-dependent structural alterations in cytochromes P-450 some of the forms were purified from young and aging rats and characterized.

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The five forms of cytochrome P-450 purified from 10 week old rats, when induction is greatest, are shown in Figure 1. Forms 1 to 5 are named on the basis of the electrophoretic mobility in SDS gel electrophoresis from fastest moving to slowest. Form b was isolated from phenobarbital pretreated rats and has the highest turnover number for benzphetamine. It is identical in mobility and catalytic activity to form "b" purified by Ryan *et al.* (1979) and thus the name Form b is retained.

Effects of aging and induction upon the level of cytochromes P-450 in the rat liver microsomes

Immunochemical analysis. The multiple forms of cytochrome P-450 were quantified by radial immunodiffusion with six different antibodies prepared to the purified forms of cytochrome P-450, F1 to F5 and F-b.

The results of the estimation of the various forms present in hepatic microsomes isolated from β-naphthoflavone or phenobarbital pretreated rats at ages of 2 weeks and 52 weeks are presented in the histograms in Figure 2. These results indicate that Form 5 is induced by β-naphthoflavone but shows no increase in rats treated with phenobarbital. The older rats show a decrease in the level of Form 5. Form 4 is present in both β-naphthoflavone and phenobarbital treated rats, but not in the control microsomes. There is a decrease in the level of Form 4 with age in β-naphthoflavone pretreated rats,

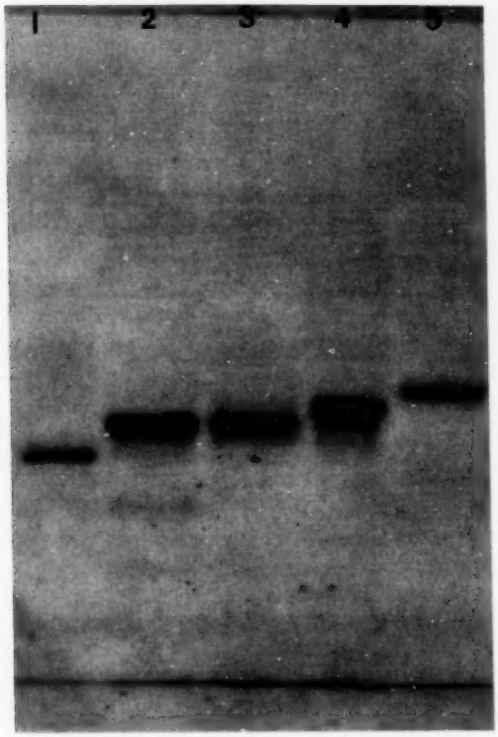

FIG. 1. SDS gel electrophoresis of the purified forms of cytochrome P-450 from hepatic microsomes of β-naphthoflavone-pretreated 2-week-old rats. A 7.5% gel system according to the method of Laemmli *et al.* was used. Lane 1 contained 5 μg of Form 1 (M_r = 47.0 K, λ_{max} = 452.5 nm); lane 2, 8 μm of Form 2 (M_r = 50.5 K,λ_{max} = 449 nm); lane 3, 8 μg of Form 3 (M_r = 51.5 K,λ_{max} = 449 nm); lane 4, 15 μg of Form 4 (M_r = 53.5 K,λ_{max} = 447.5 nm); and lane 5, 5 μg of Form 5 (M_r = 56.5 K,λ_{max} 447.5 nm).

but the variation with age of this form in phenobarbital pretreated rats was not significant. Young rats treated with β-naphthoflavone showed a high level of Form 3, but Form 3 was not detectable in the year old rats or the young phenobarbital-pretreated rats. There is a considerable amount of Form 3 present in aging phenobarbital-pretreated rats.

Form 2 is present in all of the microsomes tested, with a considerably higher amount in the young phenobarbital-pretreated rats. This form increases after phenobarbital treatment only in young rats. Form 1 is also present in all microsomes tested but appears to be increased by phenobarbital or β-naphthoflavone pretreatment in both young and aging rats.

Form b is present in phenobarbital-pretreated rats as the dominant form. This observation is in agreement with that reported by Thomas *et al.* (1979). This form also appears in aging rats after βNF pretreatment.

The method used to estimate the effects of age on the hepatic response to induction by phenobarbital and β-naphthoflavone clearly demonstrates that there is considerable variability in response depending on the specific form analyzed. These data seem consistent with the possibility of multiple controls regulating recognition or response to in-

ducers. These data must be interpreted cautiously, however, since the immunochemical method of estimation is limited by several factors. For instance, the antibody may react with apocytochrome P-450 or denatured cytochrome P-450 as well as with the native active holocytochrome P-450. The first two species are inactive for different reasons while only the last species is catalytically active. Similarly cross reactivity due to the presence of common epitopes may inflate the estimation of the amount of a form present. This method gives an estimate of increases or decreases of specific forms rather than absolute quantitation of the amounts of specific cytochromes P-450. We optimized the quantitative in so far as possible by measuring directly the diffused area of immunoprecipitate on the same gel for each rabbit antibody for all age and induction groups tested. Thus each set of measurements with replicates was internally controlled and the data averaged and presented in the histograms in Figure 2.

SDS-Gel-electrophoresis. In an attempt to visualize age-related changes in the distribution of cytochromes P-450 indicated by immunochemical estimation experiments SDS electrophoresis was performed on microsomes from young and older rats pretreated with β-naphthoflavone or phenobarbital as suggested from the work of Rikans *et al.* (1982a, 1982b).

In Figure 3A, the electrophoretic bands corresponding to Forms 3, 4, and 5 in lane A

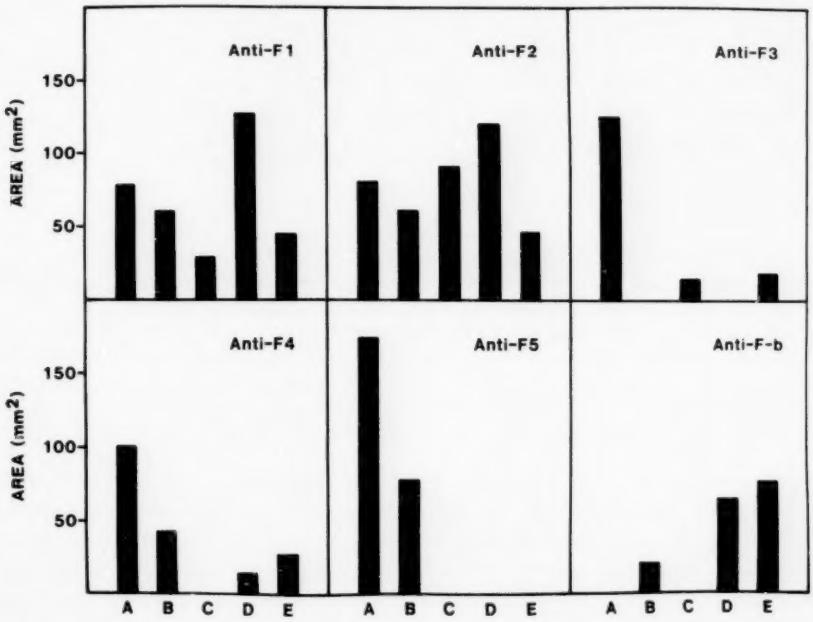

FIG. 2. Immunoquantitation by radial diffusion of the forms of cytochrome P-450 present in liver microsomes from rats of different ages. Quantitations were performed with antibodies to the purified Forms 1,2,3,4,5 from β-naphthoflavone pretreated rats and to Form b from phenobarbital pretreated rats. Group A contained 10 μg of solubilized liver microsomes from β-naphthoflavone pretreated 2-week-old rats; Group B, β-naphthoflavone pretreated 52-week-old rats; Group C, control (untreated) 2-week-old rats; Group D, phenobarbital pretreated 2-week-old rats; and Group E, phenobarbital pretreated 52-week-old rats. The area of diffusion of the immunoprecipitant was determined by subtracting the square of the internal diameter from the square of the outer diameter.

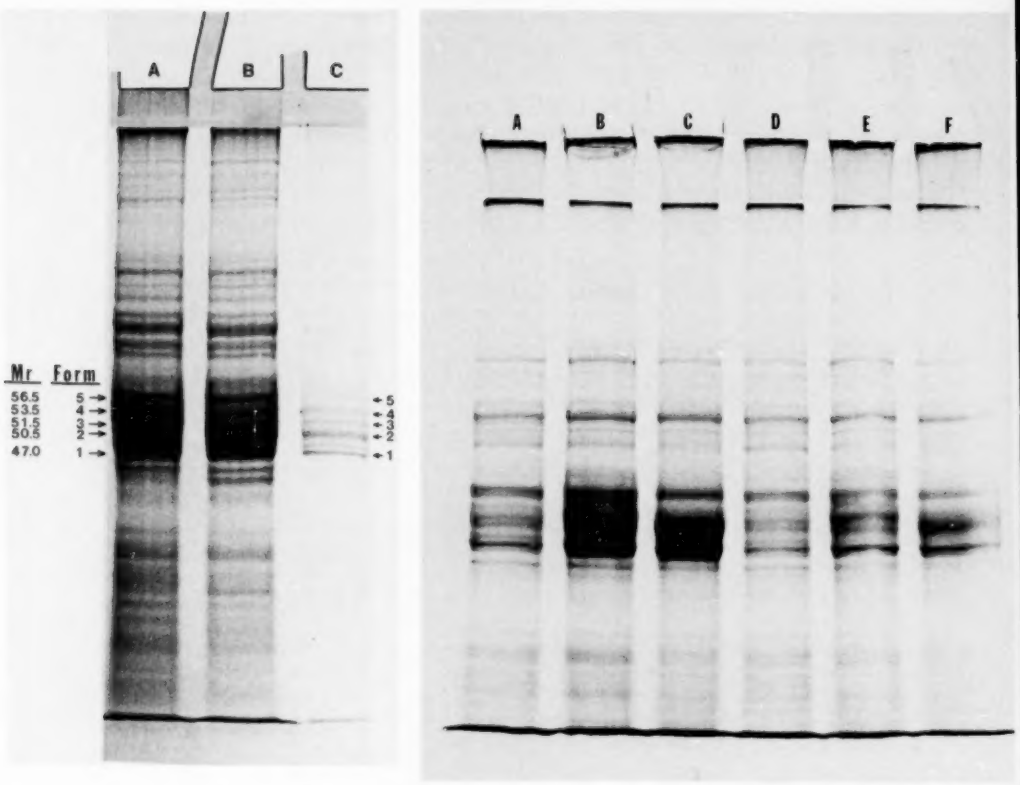

FIG. 3. A: SDS gel electrophoresis of β-naphthoflavone microsomes of different age groups Lanes *A* and *B* contained crude microsomes (20μg); *A*, microsomes from 2-week-old β-naphthoflavone treated rats; *B*, microsomes from 52-week-old β-naphthoflavone treated rats. Lane *C* contained cytochrome P-450 fraction after DEAE Sephadex A25 column chromatography of microsomes from 52-week-old rats. B: SDS gel electrophoresis of phenobarbital and control microsomes of different age groups. Lanes A–C contained crude microsomes (20 μg); *A*, control microsomes; *B*, microsomes from 2-week-old phenobarbital treated rats; *C*, microsomes from 52-week-old phenobarbital treated rats. Lanes *D–F* contained crude microsomes (10 μg); *D*, control; *E*, microsomes from 2-week-old phenobarbital treated rats; *F*, microsomes from 52-week-old phenobarbital treated rats.

(β-naphthoflavone treated 2-week-old rats) are the predominant species. Form 2 is the predominant species in the older β-naphthoflavone treated rats (lane B). The distribution of protein bands from microsomes of β-naphthoflavone-treated 52-week-old rats after chromatography on DEAE Sephadex shown in lane C exhibits a similar pattern of dominant species as seen in lane B. The results agree well with the immunodiffusion data.

As shown in Figure 3B (lanes B and E), Form 1, Form 2, and Form b seem to be the predominant species in young phenobarbital-treated rats, whereas Form b appears to be the predominant cytochrome P-450 in the older phenobarbital-treated rats. These results are also in accord with the immunochemical estimations presented in Figure 2.

These data must be interpreted with caution because the electrophoretic mobility of a form of cytochrome P-450 may be affected by the other contaminating proteins and lipids present in the microsomes due to molecular interaction or a change of denaturation cri-

teria. Also, Coomassie-Blue staining is not equally quantitative for all proteins; some peptides which stain demonstrably with silver nitrate stain only lightly with Commassie-Blue.

The resolution of the multiple forms of Cytochrome P-450 as a function of aging and induction

That aging has varying effects on the multiple forms of cytochrome P-450 is further demonstrated by separation and partial purification of cytochromes P-450 from rat liver microsomes prepared from different age groups after pretreatment with different inducers. The identification of the purified forms was performed by characterization of subunit molecular weight, CO-difference absorption maximum, and Ouchterlony immunodiffusion patterns.

In the resolution of microsomes from young rats pretreated with β-naphthoflavone, five forms were purified (Table 1, experiment A). These results are consistent with our earlier report (Lau and Strobel, 1982). In the older rats pretreated with β-naphthoflavone (Table 1, experiment B), only Forms 1 and 5 were purified to electrophoretic homogeneity. The separation was not as complete as in the preparation from the young rats (this observation of incomplete separation of multiple forms in older rats is very persistent). The interference may be due to a different content of lipids in the tissues of older rats as has been suggested by others (Rikans and Notley, 1982b). Fraction 3 contains F4 and F5, as shown by Ouchterlony immunodiffusion and SDS electrophoresis analysis.

TABLE 1. RESOLUTION, PARTIAL PURIFICATION, AND IDENTIFICATION OF FORMS OF CYTOCHROME P-450 PRESENT IN HEPATIC MICROSOMES OF 2- AND 52-WEEK-OLD RATS PRETREATED WITH β-NAPHTHOFLAVONE AND PHENOBARBITAL.

Preparation A (β-naphthoflavone – 2 weeks)

Frac.	*S.C.*	*CO_{max}*	*Ouch. Form*	*SDS-gel Form*
1	9.1	449.5	2	2
2a	2.1	449	N	3
2b	16.5	447	4	4
3	17.2	450	1	1
4	12.1	450	2,1	1
5	15.5	446	5	5

Preparation B (β-naphthoflavone – 52 weeks)

Frac.	*S.C.*	*CO_{max}*	*Ouch. Form*	*SDS-gel Form*
1	15.4	448.5	3,2,1	3,2,1
2	6.1	451	1	1
3	13.4	447.5	5 spur	5 mixed
4	10.3	446	5	5

Preparation C (Phenobarbital – 2 weeks)

Frac.	*S.C.*	*CO_{max}*	*Ouch. Form*	*SDS-gel Form*
1a	8.0	450	2,3	2
1b	6.4	450	3,2	2,3
2	9.3	449	b	b

Preparation D (Phenobarbital – 52 weeks)

Frac.	*S.C.*	*CO_{max}*	*Ouch. Form*	*SDS-gel Form*
1a	8.06	450	N	M
1b	10.3	450	2	2
2	3.51	450	1	M
3a	14.0	450	b	b
3b	9.2	449	b	M

N: Not detected
b: Form b, benzphetamine-active form
M: Multiple bands
S.C.: Specific content (nmol/mg)
CO_{max}: Absorption maxima in reduced carbon monoxide difference spectrum (nm ± 1 nm)
Ouch. Form: Immunochemical identification of the presence of specific forms of cytochrome P-450
SDS-gel: Identification of specific form of cytochrome P-450 based on molecular weight

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Complete chromatographic resolution of the multiple forms of cytochrome P-450 from control rat liver microsomes or PB-pretreated microsomes was only partially successful. However, the procedure used here is able to resolve some of the multiple forms of cytochrome P-450 in microsomes from phenobarbital pretreated rats. Form b was purified from both young and older rats, and Form 2 was purified from the young rats pretreated with phenobarbital (Table 1C, D). In general, the level of all the multiple forms tested decreases as a function of aging in rat liver microsomes. Form 5 [equivalent to Form c (Ryan *et al.*, 1979) and cytochrome P-448 (Lau *et al.*, 1982)] is exclusively induced by β-naphthoflavone regardless of the age of the rat. Form 1, which appears identical to Form a from Arochlor-induced rat liver microsomes, has been reported to show elevated activity for testosterone 7α-hydroxylation (Ryan *et al.*, 1979). Form 1 is present at a relatively higher level in microsomes from β-naphthoflavone pretreated young rats. More interestingly, Form 1 is also present in microsomes from phenobarbital pretreated and control rats at a high level.

DISCUSSION

All of the techniques employed for this work provide evidence consistent with the reported general decline in the drug metabolizing ability of the liver with age. It is also evident, however, that not all forms change to the same degree. Since the various forms of cytochrome P-450 each exhibit pronounced differences in substrate specificity, a differential degree of change in the content of specific cytochromes P-450 is consistent with variations in metabolism dependent upon the substrate used (Rikans and Notley, 1982a; Lau and Strobel, 1982). Since hepatic first pass metabolism plays so important a role in the fate of drugs, any alteration in specific forms of hepatic cytochrome P-450 may correspondingly alter the profile of metabolites (in kind as well as amount) in the circulation leaving the liver. Hence, parent drug or metabolites not normally reaching extrahepatic tissues might be presented to extrahepatic tissues and their respective drug metabolism systems due to alterations in hepatic cytochrome P-450 content.

Our data demonstrate marked changes in the content of the multiple forms of cytochrome P-450 in liver microsomes of rats as a function of age. These changes are consistent with the decreases in two forms observed in rabbits by Chiang *et al.* (1982). Recently convincing evidence has been presented by Morgan *et al.* (1985) showing sexual differentiation of cytochromes P-450 in rat liver. These authors conclude that a specific isozyme RLM_5 is the male specific 16α hydroxylase. Using different enzyme activities Kitagawa *et al.* (1985) showed that the sex difference in activities disappear in old age (24 months) due to the decline of activities in the male. Kamataki *et al.* (1985) have shown decreases with age in the content of male specific cytochrome P-450 (designated P-450 male) by immunoquantitation techniques but not of the analogous female form. Our data presented in this paper are consistent with the work of these investigators since we used male rats in our study. The work of Kamataki and Kitagawa and their colleagues serve as an effective limit on the extrapolation of our data for male rats to female rats.

Thus, our data and the work of other investigators suggest that age itself may be a marked effector of the fate of drugs in that it modifies the amount and distribution of hepatic cytochromes P-450. This is clearly not the only effect of aging on drug metabolism. Stohs *et al.* (1980) have suggested that reduced gluthatione (GSH) levels decrease in blood and that reduced glutathione levels decreased in all tissues with age. These decreases

may affect conjugation reactions although definitive data are lacking. On the other hand, our laboratory has shown (Sun and Strobel, manuscript submitted) that certain forms of cytochrome P-450 increase more in extrahepatic tissues in older rats than in young immature rats after β-naphthoflavone treatment. Liver cytochromes P-450, however, show a decreased response to inducers with age. Increased degradation may play a role in this decreased response. Thus the overall response to aging in the capacity to metabolize drugs is probably the summation of individual responses of oxidative and conjugative enzyme systems in all the various tissues and organs of the body. Since most conjugations reactions require an activated (hydroxylated) substrate it would appear that changes in the cytochrome P-450 system would exert profound effects on the final drug and drug metabolite composition of blood. Thus, age related effects on the cytochrome P-450 system in liver may lead to significant changes in the drug/metabolite composition purveyed to tissues. The age related changes in cytochrome P-450 composition and response to induction may provide at least a partial explanation for the increased sensitivity of aged patients to drug dosage observed clinically (Schmucker, 1979; Gillette, 1979).

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ENDOGENOUS PYROGEN/INTERLEUKIN-1 PRODUCTION IN AGED RATS

CAROL A. KAUFFMAN, M.D.*

Division Of Infectious Diseases, Department Of Internal Medicine,
Veterans Administration Medical Center, and University Of Michigan Medical School,
Ann Arbor, Michigan 48109

Abstract—The effect of aging on the ability of macrophages to produce endogenous pyrogen/interleukin-1 (EP/IL-1) was tested in 13 young adult (2–3 mo) and 13 aged (24–26 mo) Fischer rats using a febrile response assay. Peritoneal macrophages from aged rats produced EP/IL-1 as well as young adult rats. Decreased production of EP/IL-1 does not appear to explain the diminished febrile response seen in the elderly.

Key Words: endogenous pyrogen, interleukin-1, febrile response, macrophages, fever in aged rats

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INTRODUCTION

CLINICAL DATA suggest that elderly persons do not manifest as vigorous a febrile response as younger persons (Yoshikawa, 1983). We have used the Fischer 344 aged rat model to explore the effects of age on the febrile response. Previous experiments showed a diminished febrile response to endotoxin in aged rats, but only if they were kept in a cold environment (Tocco-Bradley, Kluger, and Kauffman, 1985). Further work revealed that the febrile response in aged rats was similar to that of young adult rats when given either intracerebroventricular or intravenous endogenous pyrogen/interleukin-1 (EP/IL-1), the protein responsible for the induction of fever (Tocco-Bradley, Singer, Kluger, and Kauffman, 1985).

Thus, it appeared that aging did not have adverse effects on the hypothalamus or on the peripheral thermoeffector mechanisms which respond to EP/IL-1. The current study assessed the effect of aging on one further aspect of the febrile response, the production of EP/IL-1 by macrophages.

MATERIALS AND METHODS

Fischer 344 male rats were housed in individual cages at 26 °C in a 12 hour light/dark photoperiod and supplied with water and rodent chow *ad libitum*. EP/IL-1 production

*Correspondence and reprint requests to: Carol A. Kauffman, M.D., Veterans Administration Medical Center, Ann Arbor, MI 48105.
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from peritoneal macrophages was studied in 13 young adult rats (2–3 months old) and 13 aged rats (24–26 months old).

Peritoneal macrophages were elicited by intraperitoneal injection of 40ml of 0.2% shellfish glycogen (Sigma, Inc.; St. Louis, MO) 3 days prior to the experiment. Macrophages were obtained by lavage of the opened peritoneal cavity with Hanks' balanced salt solution (HBSS). The number of macrophages obtained per rat ranged between 2.1×10^7 and 4.3×10^7 in the elderly rats and between 1.1×10^7 and 3.5×10^7 in the young rats. Cells were centrifuged, washed twice with HBSS and resuspended in HBSS with 10% pooled human serum, at a concentration of 2.5×10^6 macrophages/ml. Macrophage preparations were stimulated for 1 hour at 37 °C by mixing with heat-killed *Staphylococcus epidermidis* at a ratio of 20 bacteria/1 macrophage. Cells were then centrifuged, washed with HBSS and incubated for 18 hours in a 5% CO_2 atmosphere at 37 °C. Following incubation, the supernatants were collected and stored at −20 °C until assayed within the next week.

The assay used for EP/IL-1 was production of fever in healthy 4 month old Fischer 344 male rats. To measure body temperature, each rat was implanted 5 days earlier with an intraperitoneal bio-telemetry device (Mini-Mitter, Inc.; Sun River, OR) as described previously (Tocco-Bradley, Kluger, and Kauffman, 1985). Initial dose-response studies showed that a consistent and reproducible temperature response in the assay rats was obtained using 2 ml of EP/IL-1 from each donor rat (amount derived from 5×10^6 macrophages). Therefore, 2 ml of EP/IL-1 from each young adult or aged rat was injected intraperitoneally into each of 3 different recipient rats kept at 26 °C, the rat's thermoneutral zone. Body temperature was recorded every 30 minutes beginning ½ hour prior to injection and continuing for 6 hours after injection. On each day an assay was performed, one-half of the rats received EP/IL-1 and one-half received HBSS, as a control.

Results were expressed as change in temperature from baseline at each time point. Mean values ± S.E.M. for each of the 3 rats used for the assay of each sample of EP/IL-1 were obtained for each time point. Differences between the febrile responses to EP/IL-1 obtained from aged and young adult rats and between the febrile responses to EP/IL-1 and HBSS were compared using the Student *t*-test.

RESULTS

No differences were noted in the febrile responses shown by rats injected with EP/IL-1 made from macrophages of aged rats or of young adult rats (Fig. 1). The same height of fever and the same time course of the febrile response were found. Mean temperatures over six hours (ΔT_6) were similar for EP/IL-1 from aged rats ($\Delta T_6 = 0.44 \pm 0.05$ °C) and from young adult rats ($\Delta T_6 = 0.43 \pm 0.05$ °C). Fevers produced by EP/IL-1 from both aged and young adult rats were significantly different from the response to HBSS ($p < 0.001$).

DISCUSSION

Few studies have attempted to discover the cause for the decreased febrile response described in the elderly. We have used the Fischer 344 rat as a model of aging in order to explore the effect of age alone on the ability to generate a febrile response. The current data show that macrophages from aged rats produce EP/IL-1 as well as macrophages from young adult rats, thus confirming our initial studies with human monocytes in which ag-

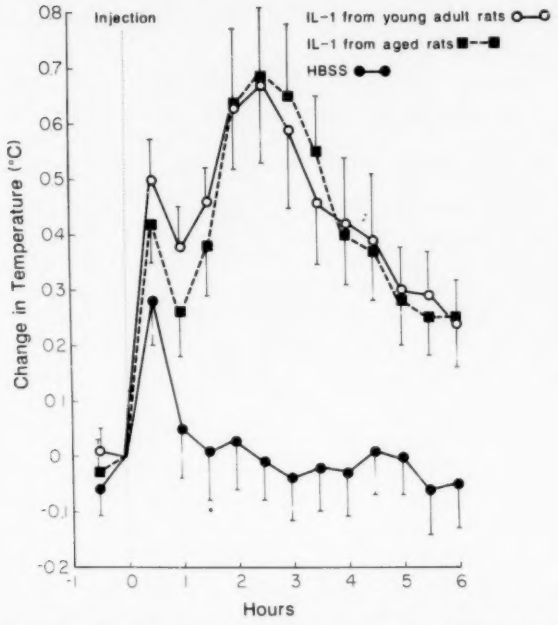

FIG. 1. Change in body temperature of rats given intraperitoneal injections of EP/IL-1 produced by peritoneal macrophages of aged rats or young adult rats or HBSS. Differences between the febrile responses to aged rat EP/IL-1 and young rat EP/IL-1 were not statistically significant. Differences between the responses to aged EP/IL-1 and HBSS and young adult EP/IL-1 and HBSS were significant ($p < 0.001$).

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ing alone did not decrease the ability of monocytes to produce EP/IL-1 (Jones, Kauffman, Bergman, Hayes, Kluger, and Cannon, 1984). These results differ from those described by Bruley-Rosset and Vergnon in mice, but their study used thymocyte proliferation rather than febrile response as the assay for EP/IL-1 and endotoxin *in vitro* as the stimulus for IL-1 production (Bruley-Rosset and Vergnon, 1984). However, Rosenberg *et al.*, using the thymocyte proliferation assay and endotoxin as the stimulus for IL-1 production in rats, obtained results similar to ours; they found that aging did not decrease EP/IL-1 production (Rosenberg, Gilman, and Feldman, 1983).

Using the Fischer rat, we have previously shown that aging does not influence the ability to respond to EP/IL-1 injected either directly into the cerebral ventricles or systemically (Tocco-Bradley, Singer, Kluger, and Kauffman, 1985). Thus, it would appear that all facets of the febrile response when tested individually function normally in the aged rat. The diminished response to endotoxin in the cold in aged rats might be related to effects of endotoxin directly on blood vessels in aging animals rather than to an effect on the EP/IL-1 mediated febrile response (Tocco-Bradley, Kluger, and Kauffman, 1985).

This study, as well as that carried out in humans, lends support to the concept that aging, per se, may not lead to a defect in the febrile response, but that other events which are common in the aged, such as malnutrition and chronic disease may be more important in diminishing the febrile response (Bienia, Ratcliff, Barbour, and Kummer, 1982; Keenan, Moldwawer, Yang, Kawamura, Blackburn, and Bistrian, 1982).

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ENDOGENOUS GABA CONCENTRATION IN CORTICAL SYNAPTOSOMES FROM YOUNG AND AGED RATS

D.D. WHEELER* and J.G. ONDO

Department of Physiology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, South Carolina 29425

Abstract—The objectives of this study are threefold: (1) to compare the γ-aminobutyric acid (GABA) concentration of a preparation of cortical synaptosomes from young male rats to that of aged rats; (2) to compare the GABA concentration in synaptosomes retained by Millipore filtration for young and aged rats; and (3) to compare the GABA concentration in the synaptosomal preparation to that in synaptosomes retained by Millipore filtration for young and aged rats. The GABA concentration is measured in cortical synaptosomes from Long-Evans rats at 2 months and 30 months of age. Concentration of the synaptosomal preparation declined significantly from 2.97 mM in 2-month animals to 2.68 mM in 30-month animals. In order to compare the results with previous studies of GABA transport, in which Millipore filters are used to separate synaptosomes from incubation medium, the GABA concentration is also measured following placement of synaptosomes on 0.65μ pore size Millipore filters. For both young and aged animals, this population of synaptosomes is found to contain GABA at a concentration more than triple that in the overall population. This is the expected result if the filtration process reduces the non-synaptosomal component of the total preparation, since GABA uptake is known to be a function of the nerve-ending component. Comparison of 2-month and 30-month synaptosomes reveals that the GABA concentration of those synaptosomes retained by the filtration process declined to a greater extent in the aged group than did the content of the overall population (from 9.33mM to 8.37mM). The results are consistent with previous studies of the aging of the GABA transporter in this preparation, in which it was found that the GABA transport capacity declined in the 30-month age group.

Key Words: aging, GABA concentration, synaptosomes, cortex, rat

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INTRODUCTION

PREVIOUS STUDIES of synaptosomal transport processes have shown that certain mechanisms are sensitive to the effects of age, while others appear to be refractory. Studies of the synaptosomal transport of 2-deoxy-D-glucose, a low affinity mechanism, showed no changes when 2-month and 30-month animals were compared (Wheeler, 1981a). However, studies in this laboratory of high affinity GABA and glutamate transport in rat cortical and hypothalamic synaptosomes have shown that alterations occur in these transport

*Address reprint requests to Dr. Wheeler at the above address.
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mechanisms as the animal ages (Wheeler, 1980a; 1982a; 1983). In kinetic studies of the sodium dependence of GABA transport in cortical synaptosomes from young and aged animals (Wheeler, 1982a), initial velocity of uptake was found to decline by as much as 22% in the aged group. However, the data were found to fit the same kinetic model (shown below) as did the data from the young group. Thus

$$\begin{array}{ccccc} & & \overset{K_4}{} & & \overset{K_5}{} \\ C & + Na \rightleftharpoons & CNa & + Na \rightleftharpoons & CNa_2 \\ + & & + & & + \\ G & & G & & G \\ & \Updownarrow K_1 & \Updownarrow & & \Updownarrow \\ & & \underset{K_2}{} & & \underset{K_3}{} \\ CG & + Na \rightleftharpoons & CNaG & + Na \rightleftharpoons & CNa_2G \\ & & k_6 \downarrow & & k_7 \downarrow \end{array}$$

C = carrier
G = GABA
Na = sodium
K_1–K_4 = dissociation constants
k_6, k_7 = translocation constants
$K_6 = k_6\,[C]t$
$K_7 = k_7\,[C]t$

$$v = \frac{\dfrac{K_6[Na][G]}{K_1K_2} + \dfrac{K_7[Na]^2[G]}{K_1K_2K_3}}{1 + \dfrac{[G]}{K_1} + \dfrac{[Na][G]}{K_1K_2} + \dfrac{[Na]^2[G]}{K_1K_2K_3} + \dfrac{[Na]}{K_4} + \dfrac{[Na]^2}{K_4K_5}} \tag{1}$$

$$v = \frac{\dfrac{K_3K_6[Na] + K_7[Na]^2}{K_2K_3 + K_3[Na] + [Na]^2}[G]}{\dfrac{K_1K_2K_3K_4K_5 + K_1K_2K_3K_5[Na] + K_1K_2K_3[Na]^2}{K_2K_3K_4K_5 + K_3K_4K_5[Na] + K_4K_5[Na]^2} + [G]} = \frac{V_a[G]}{Kt + [G]} \tag{2}$$

there was no change with age in the fundamental mechanism by which sodium, GABA, and carrier interact in the process of transport. The constants which quantitate the model were found to change with age. As a consequence, those transport parameters indicative of transport capacity were found to decline with age: (1) Hypothetical transport capability when both sodium and GABA concentrations are infinite declined by about 20% in 30-month old animals compared to 2-month animals; (2) *Va*, apparent maximal velocity of uptake, declined by about 14% at physiological sodium concentration; (3) *Jm*, rate of uptake at infinite sodium concentration and a given GABA concentration, also declined in the aged group from 15% to 20%, depending on the GABA concentration. Those parameters indicative of apparent carrier affinity for GABA were less affected by aging in the physiological range of sodium concentration. However, apparent transport affinity for sodium was found to increase somewhat in the aged group.

As a consequence of the decline in GABA transport capacity, one would predict that the concentrative capacity of nerve endings should also decline with age. Since the ability to concentrate GABA in nerve endings is integral to GABA's function as a neural transmitter (Krnjević, 1974; Roberts, Chase, and Tower, 1976), the decline in this capability with age might be expected to have serious deleterious effects on neuroregulatory mechanisms in aged animals. Thus the present study was undertaken to determine whether or

not the decline observed in the ability of synaptosomes to take up GABA is truly reflective of a decline in the ability of nerve endings to concentrate endogenous GABA. The results are consistent with predictions based on the previous *in vitro* transport studies.

METHODS

Male Long-Evans rats 2–3 months or 29–30 months old were used in this study. These rats were housed two per cage under conditions of constant temperature and controlled illumination, with *ad lib.* access to food and water. The diet was obtained from Wayne Feeds (Allied Mills, Inc., Libertyville, Ill.).

Preparation of Synaptosomes

The details of the synaptosomal preparation procedures have been published (Wheeler, 1982a). Old and young animals were handled in precisely the same manner. Animals were sacrificed by decapitation and the brains were rapidly removed and placed on glass dissection plates on ice. The cortices were then rapidly dissected free and homogenized in 20 volumes of ice cold 0.32 M sucrose. The homogenates were centrifuged for 10 min. at 1085 × g (2 °C) to remove nuclei and cellular debris. The synaptosomes were then sedimented by centrifugation at 27,000 × g for 15 min. The supernatant was discarded, and the synaptosomes were resuspended in cold sucrose.

For analysis of the GABA concentration of this suspension, duplicate 0.6 ml samples of suspension were taken and each was added to a 0.4 ml aliquot of 0.1 N HCl and saved for analysis as described below. For analysis of the GABA concentration of synaptosomes trapped by Millipore filters, synaptosomes were added to the filters by use of procedures similar to those used for transport experiments. Six-tenths ml samples were added to 11.4 ml of Krebs–Henseleit solution (30 °), mixed, added to the well of a 30-place filtration manifold fitted with a 0.65 μ pore size Millipore filter, and washed once with 5 ml of Krebs–Henseleit solution (30 °C). Duplicate samples were prepared and each filter placed in 1 ml of 0.1 N HCl. In previous transport experiments, 0.2 ml samples of synaptosomal suspension were used along with 0.45 μ pore size Millipore filters. In order to obtain sufficient GABA for reliable measurements, the sample size was tripled in these experiments to 0.6 ml. This amount of tissue was found to clog 0.45 μ pore size filters, thus 0.65 μ pore size filters were used instead.

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Measurement of Endogenous GABA Concentration

The two samples of suspension or filters from each animal were combined, centrifuged at 27,000 × g for 15 minutes to remove any debris, and the supernate saved for analysis. Duplicate 20 μl samples were assayed for GABA by a modified version of an established enzymatic technique (Jakoby and Scott, 1959; Graham and Aprison, 1966; Ondo *et al.*, 1982). GABA is converted to succinate by GABA transaminase and succinate semialdehyde dehydrogenase with the formation of equivalent moles of NADPH. Using a sensitive Perkin–Elmer 650-40 spectrofluorometer, the amount of NADPH formed in the GABA assay is measured by direct fluorescence according to the method of Lowry (Lowry, Roberts, and Kapphahn, 1957). The assay is highly sensitive, with a lower detection limit of 25 picomoles.

Intrasynaptosomal Water Content

Distributions of ^{14}C-inulin and ^{3}H-D-galactose were utilized to compute synaptosomal water content. Synaptosomes were prepared as usual. Sufficient inulin and D-galactose were then added to bring the final concentration of each to 10^{-4}M, including 2 μCi/ml ^{14}C-inulin and 5 μCi/ml ^{3}H-D-galactose. After standing in an ice bath until equilibrium had resulted (over night), 0.6 ml aliquots were added to 11.4 ml aliquots of Krebs–Henseleit solution (30 °C), mixed, and the synaptosomes separated from the buffer by filtration. Each filter was then washed twice with 5-ml aliquots of Krebs–Henseleit (0 [Na], 30 °C). Each filter was then added to 1 ml distilled water in a scintillation vial and allowed to stand for 1 hour. Scintillation fluid was then added and the sample was counted for radioactivity by liquid scintillation spectrometry. After correcting the counts for crossover, intrasynaptosomal volume was computed as the difference between the D-galactose and inulin volumes of distribution.

Statistical Analysis

A two factor analysis of variance was carried out with the use of Human Systems Dynamics' ANOVA II program and an Apple II+ computer. Significance of the difference in each mean between 2-month and 30-month animals was also tested by a *t-test*.

RESULTS

Synaptosomal Water Content

Synaptosomal water content was measured following addition of 0.6 ml aliquots of synaptosomal suspension to 0.65μ pore size Millipore filters for both young and old animals. For 2-month old animals, a mean value of 1.36 ± 0.12 (SEM) microliters of synaptosomal water per milliliter of synaptosomal preparation was obtained for 18 determinations on six animals. For 30-month old animals, a mean value of 1.34 ± 0.07 (SEM) microliters resulted. Thus, there is no difference in water content between synaptosomes from young and old rats (a *t*-test between the two groups gave a *P* value greater than 0.8). Previous measurements of the water content of nonfiltered synaptosomes gave values of 7.15 and 7.35 μl per ml of synaptosomal preparation for 2-month old and 30-month old animals, respectively (Wheeler, 1982a). Thus filtration results in the loss of much of the membrane bound water.

Statistical Analysis

The GABA concentration in synaptosomes from young and old animals is shown in Table 1. For each synaptosomal preparation, measurements were made on synaptosomes treated in two ways. First, analysis was made of the GABA concentration of 0.6 ml samples of the synaptosomal suspension, which corresponds to the synaptosomes resulting from 30 mg tissue wet weight. Second, analysis was made of the synaptosomes retained by 0.65 μ pore size Millipore filters following application of 0.6 ml of the synaptosomal suspension. Significance of the difference in GABA concentration of synaptosomes from 2-month and 30-month animals was assessed by a two factor analysis of variance (Table 2). The between factor is animal age, while the within factor is sample treatment. The difference in synaptosomal GABA concentration between the 2-month old and 30-month

TABLE 1. ENDOGENOUS GABA

Age	Concentration in mM		Ratio
	Filter	Suspension	Filter/Suspension
2 Months	9.33 ± 0.23	2.97 ± 0.07	3.15
30 Months	8.37 ± 0.36	2.68 ± 0.08	3.12

Values shown are the means ± SEM of duplicate samples from seven 2-month animals or twelve 30-month animals. Previous estimates of the water content of the synaptosomes in suspension (Wheeler, 1982a) were used to calculate the GABA concentration in the suspension, while the results described above were used to compute the concentration in synaptosomes retained by filters. GABA concentration is expressed as millimoles per liter of synaptosomal water (mM).

old animals is significant, since $P = 0.045$. The difference in concentration between synaptosomes analyzed directly from the preparation and those retained by the filter is also highly significant, since the within subjects factor (sample treatment) has a P value less than 0.001. However, there is no significant interaction of age and sample treatment ($P = 0.157$), which means that the difference between filtered and non-filtered synaptosomes occurs for both age groups.

GABA Concentration of the Non-filtered Synaptosomal Preparation

In synaptosomes from 2-month old animals, the GABA concentration is 2.97 mM (Table 1), which declines to 2.68 mM in the 30-month old group. Thus the GABA concentration is about 8% higher in synaptosomes from 2-month animals than in synaptosomes from 30-month animals. A t-test gave a P value between 0.025 and 0.01; thus this difference is significant.

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GABA Concentration in Synaptosomes Retained by 0.65 μ Filters

In the experiments on the aging of the GABA transporter, Millipore filters were utilized to separate synaptosomes from incubation medium. Thus the population of synaptosomes which contributes to the measured uptake is that fraction which remains intact on the filter. Measurements of "cell" water of both the synaptosomal suspension and of the intact synaptosomes following placement on Millipore filters showed a substantial loss of "cell" water when suspensions of synaptosomes equivalent to that used in the transport ex-

TABLE 2. ANALYSIS OF VARIANCE.

Source	SS	DF	MS	F	P
Between					
Age	3.474	1	3.474	4.559	0.045
Error	12.953	17	0.762		
Within					
Sus/Filter	320.777	1	320.777	701.919	<.001
Age × Sus/Filter	0.986	1	0.986	2.158	0.157
Error	7.775	17	0.457		

Human Systems Dynamics' ANOVA II program was run on an Apple II+ computer.

periments were placed on filters and separated from incubation medium. In order to compare the synaptosomal GABA concentration to the results of the transport experiments, it was therefore necessary to measure the GABA concentration of synaptosomes retained by the filters (Table 1). In 2-month old animals, the GABA concentration of the population of synaptosomes remaining on the filter is 9.33 mM compared to 8.37 mM for the 30-month old animals. Thus the concentration is about 12% higher in the young animals. This difference is significant ($0.05 > P > 0.025$).

Comparison of Nonfiltered and Filtered Synaptosomes

Note that the GABA concentration of the synaptosomes trapped by the filters is more than triple the concentration of the synaptosomal preparation. Thus the process of separation of the synaptosomes from incubation medium has resulted in the segregation of a fractional population with GABA concentrating capacity much greater than the overall population. This is the expected result if the loss of cell water caused by filtration is the result of the loss of a greater fraction of nonsynaptosomal cell water than synaptosomal cell water. This implies that the process of filtration concentrates the synaptosomal component. Indeed, this has been found to be the case in electronmicrographic studies of this preparation, in which it was found that the membrane bound volume of the synaptosomal suspension was only 28% synaptosomes, whereas this percentage rose to 61% when the preparation was placed on 0.65 μ pore size filters (Collings *et al.* 1986). This population is also more susceptible to the effects of age. The GABA concentration of synaptosomes retained by filters is about 12% higher in 2-month animals compared to 30-month animals, while, as pointed out above, GABA content for the overall population is only 8% higher in the 2-month age group.

DISCUSSION

From the results of previous transport studies, one would predict that the GABA concentrative capacity of nerve endings should decline with age. The present results are consistent with this prediction.

What is the basis of the decline with age in GABA concentrative capacity? Three possibilities come immediately to mind: (1) GABA transport is almost totally dependent on external sodium concentration (Martin and Smith, 1972; Martin, 1973; Ryan and Roskoski, 1977; Wheeler and Hollingsworth, 1979; Wheeler, 1980b; 1980c; 1981b; 1982b). Thus any decline with age in the ability to maintain the high external sodium concentration would adversely affect the capability of the GABA transporter, and hence lower the GABA concentrative capacity. However, this possibility could not explain the decline in transport capability with age which was seen in the transport studies, since the external sodium concentration was maintained at fixed values in those studies. (2) Maximal carrier transport capacities are dependent on both the carrier concentration and the rate constants for translocation of the loaded carrier species (see the rate equation in the Introduction). Thus a decline with age in either of these factors would decrease the ability of the nerve endings to take up GABA. Two loaded carrier species are translocated in the overall mechanism by which GABA is taken up $-CNa^{+}G$ and $CNa_2^{++}G$ (C = carrier; G = GABA). A decline with age in translocation constant (k_6 or k_7) for either of these species would reduce transport capability. Examination of the rate equation reveals that Va, the apparent maximal velocity, is also dependent on certain of the dissociation constants (K_2 or K_3). Thus,

changes in these constants with age could alter the rate of transport. (3) In previous studies, we have found that the uptake of both glutamate (Wheeler and Graves, 1983) and GABA (Wheeler, 1984) is highly dependent on the membrane potential. Thus the decline in GABA concentrative capacity could result from a decline in resting membrane potential in aged synaptosomes.

Although transport, which is regulated by membrane potential, is a major determinant of neuronal GABA concentration, it should be acknowledged that mechanisms other than transport, such as altered rate of synthesis, might also be involved in the decline in GABA concentration with age. It should also be pointed out that the interpretation of the observed differences in GABA concentration might be confounded somewhat by post-mortem changes in GABA metabolism. One such possibility is continued activity of glutamic acid decarboxylase (GAD) (Shank and Aprison, 1971; Patel, Johnson, and Balázs, 1974; Peričić, Eng, and Walters, 1978). However, we have found in previous experiments with this synaptosomal preparation that there is very little conversion of glutamate to GABA (Wheeler and Wise, 1983). Thus post-mortem differences in GAD activity would not be expected to have measurable effects on the differences in GABA levels between young and aged animals. Furthermore, the animals in both age groups were treated identically and all tissue dissections were done on ice, which should tend to minimize any post-mortem changes.

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CHANGES IN TOTAL AND MITOCHONDRIAL NUCLEIC ACID CONTENT IN AGING *CARYEDON SERRATUS* OLIVER (COLEOPTERA)

NIRMAL RAI, RENU WADHWA, AND SURAJ P. SHARMA*

Department of Biology, Guru Nanak Dev University, Amritsar-143005, India.

Abstract—Aging in the beetle *Caryedon serratus* was accompanied by a decline in total DNA content (9.28% in males and 9.79% in females). Mitochondrial DNA remained constant until the 11th day of age and then decreased gradually with advancing age in both sexes. RNA content and the RNA/DNA ratio showed similar changes in whole body as well as mitochondrial fractions. The two parameters decreased continuously and significantly ($p < 0.001$) in males whereas in females an initial rise during the reproductive period was followed by decline until death. The observations suggest that there is a loss of mitochondria in the senescent period and a decline in the total nucleic acid content and protein synthetic capacity of *C. serratus*.

Key Words: nucleic acid, mitochondria, *Caryedon serratus*, beetle, DNA, RNA

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INTRODUCTION

AGING IS characterized by a variety of deleterious changes. Theoretically all changes must be linked to the primary informational molecule, DNA and its ancillary control proteins. Medvedev (1978) proposed that the fundamental events responsible for aging changes probably can be traced ultimately to the cell nucleus. Sinan (1977) reported that changes in chromatin play a major role in age-associated deterioration of cell metabolism. Studies on the quantitative changes in DNA and RNA can be potentially useful in providing information concerning changes in cell synthetic capacity and number with age (Sohal, 1985). Available data on age-associated variations in nucleic acid content are limited and contradictory. The total DNA content has been reported to remain constant throughout the imaginal life in *Aedes aegypti* (Lang *et al.*, 1965), in *Drosophila melanogaster* (Balazs and Haranghly, 1965), and in *Musca domestica* (Yale and Ballard, 1965). Blevins (1973) reported a decrease in *A. aegypti* DNA content up to the 5th day. However decreases in nucleic acid content and RNA/DNA values have been reported in both sexes in *D. melanogaster* (Samis *et al.*, 1971), *Lucilia sericata* (Ring, 1973), *Triatoma infestans* (Mello and Raymundo, 1977), *Callosobruchus maculatus* (Sharma et al., 1984) and *D. bipectinata* (Wadhwa and Sharma, 1984), suggesting that physiological aging is accompanied by a possible decrease in protein synthetic potential. Massie *et al.* (1975, 1981) reported a

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striking loss of mitochondrial DNA in *D. melanogaster* while the nuclear DNA content remained constant. This is in agreement with the view of Miquel *et al.* (1983) that the site of senescent injury is mitochondrial DNA. The present work was performed to investigate changes in the total nucleic acid as well as in the mtDNA of aging *C. serratus*.

MATERIALS AND METHODS

Rearing and Maintenance

Biological samples of *C. serratus* Oliver (Bruchidae: Coleoptera) were reared on the seeds of *Tamarindus indica* L. at 28 ± 2 °C and in a relative humidity of 70–80%. Cultures of different age groups were maintained in 1.5 liter glass jars with their openings covered with fine muslin cloth. Males and females were separated on the basis of external morphological characters (Arora, 1977). Experiments were performed in triplicate on different age groups (1, 3, 5, 7 . . . 17 days) of bruchids. All preparative operations were performed at 0–4 °C.

The mean and maximum life spans at 28 ± 2 °C are 16.72 and 24 days for males and 15.42 and 22 days for females respectively. The adults of *C. serratus* do not feed during their life time and thus can be regarded as a semiclosed system like silkworm moths (Osanai and Kikuta, 1981).

Preparation of whole body homogenates

The bruchids of different age groups were immobilized by cooling at 4 °Cand 10% (w/v) homogenates were prepared by crushing 200 mg (approximately 10 individuals) in 2.0 ml of cold double distilled water. This was centrifuged at 3,000 rpm for 15 minutes in an R8C Laboratory Centrifuge (Remi).

Isolation of Mitochondria

Mitochondria were isolated according to the procedure described by Van den Bergh (1967) and Bulos *et al.* (1972) with these modifications. The isolation medium consisted of, 0.25 ml sucrose, 0.15 M potassium chloride and 0.1 mM Ethylenediamine-tetrachloric acid. The pH of the medium was adjusted to 7.4 at 0 °C with 0.1 M potassium chloride. Two ml of 20% homogenates prepared in isolation medium were centrifuged at 3,000 rpm for 5 minutes. Mitochondria were sedimented from the supernatant fluid by centrifugation at 16,000 rpm for 10 minutes at −4 °C using a K-70 (Janetzki) centrifuge. The mitochondrial pellets obtained were suspended in 2.0 ml of cold double distilled water.

Freshly prepared whole body homogenates and mitochondrial suspensions (2 ml each) were treated separately with 3.0 ml of cold 10% trichloroacetic acid. The mixture was centrifuged and the supernatant discarded. Lipoidal compounds were removed by treating the sediments with 95% ethanol. Nucleic acids were extracted by suspending the residue in 2.5 ml of 0.5 N perchloric acid (PCA), heating the suspensions for one hour in an 80 °C water bath and then centrifuging this preparation for 5 minutes at 3,000 rpm. The sediments were resuspended in 2.5 ml of 0.5 N PCA and then heated to 80 °C for 30 minutes. The pooled supernatants contained nucleic acids which were estimated colorimetrically using orcinol for RNA and the diphenylamine reaction for DNA following the method of Schneider (1957). Standard curves were prepared using different concentrations of deoxy-

TABLE 1. CHANGES IN TOTAL NUCLEIC ACID CONTENT IN AGING *C. serratus*.

Age in days	*μg DNA/100 mg tissue ± S.D.*		*μg RNA/100 mg tissue ± S.D.*	
	Male	*Female*	*Male*	*Female*
1	98.46 ± 3.52	98.34 ± 2.32	118.42 ± 1.96	126.10 ± 2.30
3	99.59 ± 1.21^{NS}	102.19 ± 2.96^{c}	114.77 ± 1.13^{b}	134.29 ± 3.79^{c}
5	96.26 ± 2.07^{b}	100.42 ± 2.98^{NS}	96.49 ± 2.40^{a}	141.81 ± 3.58^{NS}
7	95.95 ± 3.03^{NS}	100.73 ± 2.79^{NS}	94.30 ± 3.40^{NS}	148.04 ± 3.86^{NS}
9	92.80 ± 1.42^{b}	98.65 ± 3.85^{NS}	84.07 ± 1.13^{a}	186.41 ± 3.67^{a}
11	91.23 ± 0.97^{NS}	94.06 ± 1.85^{c}	83.69 ± 0.89^{NS}	150.59 ± 1.79^{a}
13	89.34 ± 1.95^{NS}	92.79 ± 1.42^{NS}	83.69 ± 0.89	105.26 ± 3.92^{a}
15	89.02 ± 1.86^{NS}	89.97 ± 1.54^{c}	79.31 ± 1.65^{a}	86.99 ± 2.66^{a}
17	89.34 ± 1.54^{NS}	88.71 ± 2.07^{NS}	72.73 ± 2.16^{a}	72.83 ± 2.99^{a}

n = 6
NS = Nonsignificant
a = $p < 0.001$ highly significant
b = $p < 0.01$
c = $p < 0.05$

ribose and ribose sugars. Statistical analysis was carried out by applying the students *t*-test.

RESULTS

Variations in the nucleic acid content with age in both total and mitochondrial fractions are given in Tables 1 and 2. Total DNA content (μg/100 mg tissue) decreased with advancing age in males and females from 98.46 ± 3.52 to 89.34 ± 1.54 and 98.34 ± 2.32 to 88.71 ± 2.07 respectively. The percent decrease was comparable in both sexes, being 9.28% in males and 9.77% in females. A significant ($p < 0.05$) increase was observed on the 3rd day in the DNA content in both the females only. Mitochondrial DNA was almost

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TABLE 2. CHANGES IN MITOCHONDRIAL ACID CONTENT IN AGING *C. serratus*.

Age in days	*μg DNA/100 mg tissue ± S.D.*		*μg RNA/100 mg tissue ± S.D.*	
	Male	*Female*	*Male*	*Female*
1	11.48 ± 0.71	11.32 ± 0.84	7.02 ± 0.54	5.30 ± 0.45
3	11.48 ± 0.71^{NS}	11.54 ± 0.71^{NS}	6.80 ± 0.37^{c}	10.05 ± 0.83^{a}
5	11.96 ± 0.77^{NS}	12.11 ± 1.10^{NS}	6.69 ± 0.18^{b}	13.71 ± 0.92^{a}
7	11.80 ± 0.52^{NS}	11.32 ± 1.09^{NS}	6.47 ± 0.51^{NS}	14.25 ± 0.69^{c}
9	11.64 ± 0.77^{NS}	11.48 ± 0.71^{NS}	6.39 ± 0.83^{NS}	14.46 ± 0.83^{NS}
11	11.44 ± 0.71^{NS}	11.17 ± 1.10^{NS}	5.30 ± 0.83^{a}	18.46 ± 0.83^{a}
13	9.44 ± 1.58^{c}	8.65 ± 0.71^{a}	5.12 ± 0.57^{NS}	17.73 ± 0.45^{a}
15	6.95 ± 0.72^{a}	6.10 ± 0.48^{a}	4.75 ± 0.59^{b}	11.69 ± 0.57^{a}
17	6.45 ± 0.93^{NS}	4.81 ± 0.64^{b}	2.92 ± 0.70^{a}	8.70 ± 0.72^{a}

n = 6
NS = Nonsignificant
a = $p < 0.001$ highly significant
b = $p < 0.01$
c = $p < 0.05$

TABLE 3. CHANGES IN TOTAL AND MITOCHONDRIAL PROTEIN SYNTHESIZING CAPACITY IN AGING *C. serratus*

Age in days	μg RNA/μg DNA			
	Male		Female	
	Total	Mitochondrial	Total	Mitochondrial
1	1.20	0.61	1.28	0.47
3	1.15	0.59	1.31	0.87
5	1.00	0.56	1.41	1.13
7	0.98	0.55	1.47	1.25
9	0.91	0.55	1.89	1.26
11	0.92	0.46	1.60	1.65
13	0.94	0.54	1.13	2.05
15	0.89	0.68	1.06	1.92
17	0.81	0.45	0.83	1.81

constant in both sexes during the first 11 days of life followed by a significant gradual decrease with age (11.48 ± 0.71 on the first day to 6.45 ± 0.93 on the 17th day in males and 11.32 ± 0.84 to 4.81 ± 0.64 in females). The females had a higher content of total RNA as well as of mitochondrial RNA (μg/100 mg tissue) than the males at all comparable age groups except for mtRNA for one day old males. In males, total as well as mtRNA declined continuously and significantly ($p < 0.001$) with age (1 to 17 days) from 118.42 ± 1.96 to 72.73 ± 2.16 and from 7.02 ± 0.54 to 2.92 ± 0.70, respectively. In the females an initial rise in total RNA was observed from 126.10 ± 2.30 on the first day to 186.41 ± 3.67 on the 9th day and in mitochondrial RNA from 5.30 ± 0.48 on the first day and 18.46 ± 0.83 on the 11th day followed by a decline to 72.83 ± 2.99 and 8.70 ± 0.72 on the 17th day respectively. The total RNA/DNA value declined continuously (1.20 to 0.84) in males. The mitochondrial ratio also showed a continuous decline except for a slight increase on the 13th–15th day. In females, the total RNA/DNA value showed an increase until the 9th day (1.28 to 1.89) and the mitochondrial value up to the 13th day (0.47 to 2.05) after which it declined until their death (Table 3).

DISCUSSION

The present investigation on the aging of *C. serratus* suggests that the difference in the changes of DNA content in the two sexes is indicative of their differential growth rate. The decline in total DNA with age in either sex may be either due to cell loss (Lang *et al.*, 1965; Blevins, 1973), loss of euchromatin (Mello and Raymundo, 1977), inadequacies in DNA repair (Strehler and Chang, 1979) or loss of mtDNA during isolation (Inamdar *et al.*, 1974). Lang *et al.* (1965) and Ring (1973) reported that RNA content is a measure of potential rate of protein synthesis and the RNA/DNA ratio an index of the protein synthesizing capacity. In males the total and mitochondrial RNA as well as the RNA/DNA ratio decreases continuously with age suggesting a decrease in protein synthesis with age in both whole body and mitochondria. The decrease in RNA content may be due to the reduction in RNA synthesis or to uncompensated degradation of some species of RNA. This has been reported earlier to occur in *D. melanogaster* (Samis *et al.*, 1971), *L. sericata* (Ring, 1973) and in *A. aegypti* (Blevins, 1973). Maximum RNA content and RNA/DNA value in one day old males suggests that males are fully mature at emergence. The two parameters increased in females coinciding with the reproductive period, indicating an in-

creased protein synthesis to meet reproductive demand (ova production) (Samis *et al.*, 1971; Sharma and Sharma, 1979; Sharma *et al.*, 1984 and Wadhwa and Sharma, 1984). The females had a higher RNA content and RNA/DNA values than males at all comparable ages, probably due to their ability to synthesize and retain more proteins, which has also been reported earlier by Chen and Levenbook (1966) and Blevins (1973). Mitochondrial DNA content remained almost constant until the 11th day in both sexes, followed by a gradual and significant decrease until death. This may be due to either a decrease in mitochondria number or breakage of mitochondria during isolation because of increasing fragility in old organisms (Inamdar *et al.*, 1974). If breakdown of mitochondria is the cause, then the mitochondrial contents will be added to the soluble fraction and this should increase the total nucleic acid content. But it is seen that total DNA, RNA and RNA/DNA values also decline with age. Thus, the decrease in the mitochondrial nucleic acids may be attributed to a loss of mitochondria in old age. Massie *et al.* (1975) observed a 65% loss of mt DNA in *D. melanogaster* with age. Bailey and Webster (1984) also reported a decrease in mitochondrial protein synthesis, number (30%) and translational capacity in *D. melanogaster* and mice. Likewise, many investigators have reported a decrease in the number and an increase in morphological alterations with age for mitochondria from several different sources.

In conclusion there is a loss of mitochondria during the senescent period of *C. serratus*. Deteriorative changes occur in total nucleic acid content and protein synthesizing capacity which leads the organism towards physiological decline with advancing age.

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THE REGULATION OF BLOOD SUGAR IN THE AGING BLOWFLY, *PHORMIA REGINA*

CAROL M. ANELLI and STANLEY FRIEDMAN

Department of Entomology, University of Illinois, Urbana, Illinois, 61801

Abstract—The adult blowfly, *Phormia regina*, reveals a substantial homeostatic capability when examined for its ability to regulate externally administered additions to its blood sugar. As well, results of experiments done at intervals throughout the entire adult life of the fly show that there is no decrease over time in the efficiency of operation of the complex system involved in the control of the blood sugar level. Taken together with other independent investigations of *Phormia* metabolism, the research indicates that this animal, in contrast to the rat, possesses a number of physiological systems which embody regulatory processes that may be only marginally subject to the normal "aging" process.

Key Words: blood sugar, *Phormia regina*, aging in blowflies, stress response

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INTRODUCTION

IDEAS WHICH have been developed to account for the molecular determinants of senescence and death in animals derive from broadly diverse theories based either on stochastic processes or programmed activities. Conceptual differences notwithstanding, both classes of theory consider each physiological system in the organism to be subject to an independent, time-linked decay, denoted as the "aging" of that system (Rosen, 1981). However, characteristic of theories of programmed senescence is the idea that certain of the important regulatory systems are more highly temporally conserved, remaining relatively unchanged throughout life. At the end of such a program an unknown and un-named (neuroendocrine?) destruct mechanism delivers the death blow.

The stochastic theories have the following unfortunate corollary: What is called the "aging process" is so complex and environmentally dependent (each declining unit being modulated by external stress) that expectations of making fundamental changes in species longevity by manipulation of any single system are negligible. The theories embodying programming do not subscribe to the above, but have their own problem of generalization: Each species has its own chronology, so that an understanding of the timing of events in one organism may have no validity in another.

One of the important physiological parameters studied in aging mammals is the deterioration in flexibility of the homeostatic response over time, particular hormone-enzyme interactions being used to model and generalize this activity. A number of

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experiments have been done which demonstrate that hormonal regulation of enzyme function is greatly modified with age (Adelman *et al.*, 1981), and there are even indications that aging extends to effects on the transporters of some of the molecules active at synaptic junctions and involved with homeostatic control (Wheeler, 1980). It is information of this nature which has made the general "error" theories the more attractive (Hahn, 1970) of those presently under investigation in mammals.

In an effort to examine the question of whether parallels may exist between the events involved in the aging of insects and mammals, we have made a cross-sectional study of a stress response in the adult blowfly, *Phormia regina.* The research consisted of an analysis through time of the regulatory ability of the complex system concerned with the maintenance of constancy of the blood sugar level, and the results of the investigation are the subject of this brief paper.

MATERIALS AND METHODS

Flies used in this study were taken from a laboratory colony of *Phormia regina* (Meig.) and reared through the pupal stage according to procedures previously described (Friedman, 1967). Sometime prior to adult emergence, puparia were placed in a 1 m^3 cage, and 24 hours after emergence began (the end of day 0), all remaining puparia were removed. Adult flies were maintained at 25 °C under constant light and relative humidity (50–55%), and a 10% sucrose solution was made available *ad libitum.* Sugar solutions were replaced at regular intervals.

38 to 40 hours before each experiment, a number of flies were randomly captured and removed from the cage and each male was placed in an individual glass housing vial (2.5 cm diam. × 5.5 cm) lined with plastic screen and capped with a stainless-steel screen. (In an effort to maintain a normal sex ratio in the cohort cage, females captured at this time were discarded.) A shell vial containing 10% sucrose and plugged with cotton soaked in the same solution was inserted into the screen wire cap of each housing vial and the flies were allowed to feed *ad lib.* for 24 hours, after which the sucrose solution was removed and replaced by distilled water for an additional 12–14 hours. Throughout this pre-experimental period, the flies were kept under the same constant conditions as the cohort cage.

Trehalose Tolerance Test

A 2 μl capillary tube drawn to a point and calibrated to deliver a 1 μl sample was attached to a mouth pipet and used to inject solutions into the hemocoel of CO_2-anesthetized animals. Injections were made through the intersegmental membrane between the third and fourth ventral abdominal segments. Adult flies received either 500 μg trehalose dissolved in 1 μl distilled water, or 1 μl distilled water (Chen and Friedman, 1977). Ten minutes after injection, a 0.5 μl blood sample was collected from the dorsal sinus and the flies were either neck ligated with #6.0 silk, or handled as if being prepared for ligation. Upon completion of sampling, flies were returned to the housing vials and kept under the usual constant conditions for 4 hours, after which a second 0.5 μl blood sample was taken from the neck region. During the 4 hour interval the flies were allowed neither food nor water.

Measurement Of Blood Sugar Levels

The hemolymph trehalose concentration was determined for each blood sample according to a previously described method (Chen and Friedman, 1975). The glucose concentra-

tion in the hemolymph of adult *Phormia* is typically no more than 10% of the trehalose concentration; in these experiments, glucose levels did not significantly differ from those measured in unchallenged flies and, therefore, will not be reported.

RESULTS

In order to stress the flies at intervals over their adult life span, which in this strain of *Phormia* is 24–27 days, use was made of a trehalose tolerance test. Adults of various ages were injected with 500 μg of trehalose (an amount equivalent to slightly more than the average total body trehalose in these animals [Anelli and Firedman, in press]), and were assayed for the ability to restore the normal blood trehalose level within a limited (4 hour) time period.

The brain and associated corpus cardiacum (a neurosecretory structure located in the insect head) have been shown to be the sources of the hormones involved in the regulatory process (Friedman, 1967; Chen and Friedman, 1977). (The same studies have demonstrated that the corpus allatum, a gland found in close proximity to the corpus cardiacum, which secretes the juvenile hormone precursor and is known to have a number of metabolic influences, is ineffective in stimulating either an increase or decrease in blood trehalose levels when carbohydrate stress is placed upon these insects.) In light of these findings we examined the effects of neck ligation, i.e., cutting off the sources of these hormones, on the regulation of blood trehalose.

The results of our experiments are depicted in Table 1. It may be seen that the level of blood trehalose remains generally stable in untreated insects throughout adult life. In flies which are neck-ligated the ability to maintain constant trehalose levels is somewhat diminished over a period of 4 hours, although in only a single case do even the combined traumas of H_2O injection and ligation result in a significant change from the norm. Table 1 also permits the observation that regulation stems almost totally from neurosecretory elements in the head, since animals which are injected with massive amounts of trehalose and subsequently neck ligated show practically unchanged, elevated levels of sugar in the blood 4 hours later. Finally, it is in insects which have been injected with trehalose and untreated thereafter that we are able to see the capacity for regulation. It is notable that in those animals the ability does not diminish up to the time that they are close to the end of their life span, since there are no obvious increases in variance among animals of more advanced age.

Our data also show that the blood volume of the fly is held constant through its entire life span, since injection of excess trehalose results in a consistent increase in blood trehalose concentration within 10 minutes after injection, within each population and at every age analysed. Studies using injection of ^{14}C-labelled dextran to determine blood volume corroborate this finding (Anelli and Friedman, unpublished).

DISCUSSION

The strain of *Phormia regina* used in these experiments has been bred in this laboratory for a number of years, and it has been recently demonstrated that there is a significant loss of isozyme heterogeneity in the population compared to newly captured flies (Berlocher and Friedman, 1981). However, the average life expectancy of the caged adult population is 24–27 days, similar to that of the newly caged "wild" strain from this area. Experiments described in the foregoing section indicate that during that period of time there is little or

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TABLE 1. TREHALOSE TOLERANCE TEST: EFFECTS OF NECK LIGATION AND AGE ON BLOOD TREHALOSE CONCENTRATION.

		Untreated		*H_2O injection, neck ligation*			*Trehalose injection, neck ligation*			*Trehalose injection, no ligation*		
				Time post injection			*Time post injection*			*Time post injection*		
				10 min.	*4 h.*		*10 min.*	*4 h.*		*10 min.*	*4 h.*	
Expt.	*Age (days)*	*(ug/ul ± SD)*	*(n)*	*(ug/ul ± SD)*		*(n)*	*(ug/ul ± SD)*		*(n)*	*(ug/ul ± SD)*		*(n)*
1	5	–	–	19.3 ± 7.8	16.1 ± 8.7	(8)	45.8 ± 8.3	36.6 ± 7.4	(8)	55.8 ± 6.0	26.9 ± 3.0c	(8)
	9	–	–	24.4 ± 8.6	24.8 ± 13.1	(8)	60.4 ± 7.1	59.2 ± 14.4	(10)	58.8 ± 8.6	30.8 ± 8.8c	(10)
	15	39.4 ± 2.1	(5)	32.3 ± 6.0	38.1 ± 8.6	(10)	56.0 ± 7.3	54.1 ± 8.8	(10)	57.5 ± 8.5	42.7 ± 12.4a	(7)
	21	38.5 ± 11.7	(6)	28.5 ± 4.4	41.8 ± 7.8a	(7)	56.6 ± 8.4	65.7 ± 11.2	(10)	59.0 ± 9.8	55.1 ± 12.6	(10)
	26	25.7 ± 8.6	(7)	26.9 ± 5.5	29.3 ± 10.6	(7)	59.8 ± 6.4	55.0 ± 9.2	(9)	60.3 ± 8.2	40.0 ± 15.2c	(10)
2	5	–	–	14.6 ± 6.2	11.9 ± 6.6	(8)	53.2 ± 7.4	49.1 ± 7.8	(10)	50.4 ± 10.7	31.8 ± 13.3a	(10)
	11	33.2 ± 6.4	(6)	25.3 ± 3.9	28.2 ± 6.6	(10)	57.9 ± 6.2	56.6 ± 4.8	(10)	59.0 ± 9.7	35.3 ± 9.5a	(10)
	17	25.0 ± 11.0	(6)	28.3 ± 8.4	25.5 ± 9.5	(9)	64.2 ± 9.9	64.0 ± 9.1	(10)	56.5 ± 9.0	44.6 ± 16.2	(10)
	26	29.2 ± 13.0	(6)	32.8 ± 11.8	41.3 ± 17.6	(9)	66.8 ± 8.8	64.2 ± 8.0	(10)	66.6 ± 9.6	58.2 ± 9.5	(10)
3	5	29.5 ± 6.8	(6)	–	–	–	65.1 ± 12.5	53.9 ± 10.2	(10)	60.2 ± 12.1	33.6 ± 7.5c	(10)
	11	39.8 ± 14.2	(6)	34.3 ± 13.4	–	(6)	72.2 ± 8.5	75.0 ± 11.5	(10)	66.3 ± 13.3	49.5 ± 11.8a	(9)
	16	37.1 ± 6.2	(6)	32.5 ± 6.3	38.8 ± 13.1	(6)	69.0 ± 5.3	61.3 ± 9.9	(9)	69.8 ± 9.7	46.4 ± 10.0b	(9)
	21	31.8 ± 9.1	(5)	31.1 ± 11.7	–	(8)	76.8 ± 3.2	71.2 ± 5.5	(8)	73.0 ± 13.8	52.3 ± 14.2a	(7)
4	16	37.3 ± 13.0	(6)	–	–	–	64.6 ± 6.7	59.1 ± 9.0	(7)	69.5 ± 9.6	42.9 ± 22.7a	(8)
	25	39.1 ± 5.4	(6)	30.0 ± 10.6	–	(9)	57.9 ± 9.0	61.7 ± 12.5	(10)	60.1 ± 10.0	42.0 ± 11.1a	(11)

Trehalose injection: 500 ug/fly except Expt. 1 = 375 ug/fly.
Significance test: a = $p < 0.05$; b = $p < 0.002$; c = $p < 0.001$. All other not significant. Comparisons are intragroup: 10 min. *vs* 4 h.

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no decrease in efficiency of the homeostatic systems responsible for maintenance of the blood sugar level.

The regulatory system is complex, involving hyper- and hypotrehalosemic hormones (both originating in neurosecretory elements in the head), a hydrolytic enzyme, and a number of other enzymes leading by interlocking pathways to the synthesis of both glycogen and trehalose; it may, as well, encompass membrane transport regulation (Friedman, 1978).

Several lines of evidence suggest that, of the two hormones, the hypotrehalosemic may play the more significant role in blood sugar homeostasis. Experiments have shown that elevation of blood trehalose levels *via* treatment with corpus cardiacum extracts is achieved only after starving flies for 24–48 hours (Friedman, 1967), at which time all of the stored trehalose would be expected to have been used, leaving fat body glycogen as the only major source of carbohydrate available for trehalose synthesis. (It is known that the ultimate target of hypertrehalosemic hormone action is glycogen phosphorylase.) However, when flies are placed on a normal feeding regimen, extirpation of corpora cardiaca results in hypertrehalosemia (Chen and Friedman, 1977), and, as shown in the present study, flies stressed with an abnormally high trehalose load are able to effect near normal blood levels within a relatively short time period. Both of these findings support the view that the hypo- hormone acts as a continuous modulator, whereas results of the studies with the hyper- hormone indicate that it may act only during periods of low trehalose availability.

Although experiments have not been done to test the efficiency of operation of each part of the regulatory system, and whether compensatory action has been taken by any to rationalize a failure of any other, the overall effect is far from that exhibited by the large number of systems examined in mammals, all of which seem to show the signs of aging typified by an increase in the time of response to perturbation.

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This apparent lack of "aging" parallels that of another important system in *Phormia*, i.e., the oxidative pathway of the flight muscle mitochondria. In an investigation taken up to answer questions concerned with the effects of age on biochemical reactions in the flight machinery, it was found that there were no changes in mitochondrial function and only small decreases in certain enzymes of the glycolytic pathway in senescent muscle (Hansford, 1978).

As well, recent studies on adults of two species of *Drosophila* have revealed [in contrast to a previous investigation (Miquel, 1971)], that senescence is not accompanied by fat body deterioration (Roberts, *et al.*, 1985). Our own limited observations on *Phormia* bear this out (Anelli and Friedman, unpublished). The adult fat body appears robust until the animal is practically moribund, at which time the tissue almost disappears and blood levels of trehalose decrease precipitously. However, even these physiologically decrepit flies may appear close to normal for a further short period (up to a day), in other aspects of behavior, such as escape and walking.

Thus, in *Phormia* as well as *Drosophila* species, certain important functions are almost entirely conserved, being maintained at a level approaching the efficiency of youth until the adult is almost moribund. While there are many possibilities remaining as to the cause of death, three independent studies have now confirmed the fact that the general aging process does not affect a number of the major regulatory systems in cyclorraphous flies. Perhaps this evidence asks us to focus more sharply on the question of a destruct system in these animals.

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LETTERS TO THE EDITOR

To the Editor:

Experimental Gerontology recently published a paper co-authored by N.G. Abraham, R.D. Levere and M.L. Freedman (**20:** 277, 1985) which reported studies concerning liver heme and drug metabolism in Sprague–Dawley rats of varying age. The work on heme metabolism is both novel and interesting, and germane to the problem of altered metabolism of xenobiotics in senescent organisms because of its role in the synthesis of cytochrome P-450. However, I express concern for the purportedly original observation that one of the components of the hepatic mixed-function oxidase system, as well as two enzyme activities that these components specify, are equally responsive to induction in young and old animals. This observation was reported over ten years ago in the pages of *Experimental Gerontology* (Baird *et al.*, **10:** 89, 1975; Birnbaum and Baird, **13:** 299, 1978; Birnbaum and Baird, **13:** 469, 1978). Additionally, Gold and Widnell (*Biochim. Biophys. Acta* **334:** 75, 1974) reported the absence of an age-related difference in reversibility of aging changes in glucose-6-phosphatase, another microsomal enzyme present in rat liver. Finally, Abraham *et al.* have virtually ignored a tremendous body of revelant knowledge now present in the literature, especially from the laboratories of Lora Rikans, Douglas Schmucker and Arlan Richardson, as well as the seminal studies carried out by Ryuichi Kato.

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Our laboratory also made the observation that age-related changes in a variety of hepatic mixed-function oxidases are readily observed, often in the absence of consistent age-related alterations in cytochrome P-450 content or in NADPH-cytochrome *c* (cytochrome P-450) reductase activity. We suggested (Birnbaum and Baird, *Exp. Geront.* **13:** 469, 1978) that this observation may reflect organizational or structural changes in membranes ("Senescent changes in the membrane environment and hence the organized structure of the mono-oxygenase system may result in altered function.").

We also suggested that these changes may involve specific alterations in one or more of the forms of cytochrome P-450. However, we could demonstrate no readily apparent qualitative or quantitative changes in the 3 distinct forms (now at least 10 in rat liver) of cytochrome P-450 known to exist at that time. Thus, the suggestion in the paper by Abraham *et al.*, that their findings could result from a change in the microsomal membrane, or reflect selective deficiencies in one of the cytochrome P-450 species is neither novel nor original, looks remarkably like earlier suggestions, and should have been properly referenced as such.

My interest in illuminating these deficiencies is not at all totally without self-interest. However, these points are raised to also enhance the responsibility of those now working in the field to become cognizant of previous work by others.

MALCOLM B. BAIRD
Masonic Medical Research Laboratory
Utica, NY 13504

5 March 1986

To the Editor:

We are writing in response to Dr. Malcolm B. Baird's recent letter to you stating that we inadvertently omitted reference to his and other investigators' previous work on cytochrome P-450 in old and young animals. This omission was unfortunate and we appreciate his bringing this and other facts to our attention.

However, I would like to point out that the major thrust of our paper was that in spite of a decrease in the rate-limiting enzyme of heme synthesis, δ-amino-levulinic acid synthase, and an increase in the rate-limiting enzyme of heme degradation, heme oxygenase, microsomal heme and cytochrome P-450 content were maintained. Our major conclusion that in the senescent rat hepatic heme utilization and degradation are only loosely coupled to heme production is in no way related to Dr. Baird's previous work. It appears to us that alternate source(s) of heme are available in the old animals which was, to our knowledge, properly referenced to others who have had the same conclusions. Furthermore, in our studies we showed that ALA-dehydratase was unchanged in old rats. We also reported studies showing induction and inhibition of ALA-synthase and heme oxygenase in young and old animals which in no way depended upon Dr. Baird's work. Similarly, our work with the heavy metal cobalt showed that in the old rat there was significant induction of heme oxygenase at a dose that had no effect in the young animal. This also did not depend upon previous work in Dr. Baird's laboratory.

Our laboratories over the past 20+ years have been involved in heme synthesis and heme degradation and the relationship of these to heme-containing proteins. For many years we worked with developing erythrocytes and hemoglobin as the heme-containing protein. Only recently have we been looking at liver cells and cytochrome P-450 and we were obviously not aware of work done approximately ten years ago. Recent studies in the literature have also not mentioned Dr. Baird's work and we did not perform a computer search. Certainly in the future we will be more diligent in our references.

Nader G. Abraham
Richard D. Levere
Michael L. Freedman
New York Medical College
New York, NY 10016

9 *April* 1986

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ANNOUNCEMENT

The Third Tokyo Symposium on Liver and Aging

THE THIRD Tokyo Symposium, *Liver and Aging*, will be held in Tokyo, Japan on August 20 through 22, 1986 with additional coverage of *Liver and Brain*. The primary objective of the symposium is to discuss the interactions and functional alterations of these two organs as they are affected by aging. The symposium will be limited to about 100 participants. The total number of speakers will be less than 30.

Though participation will generally be limited to predetermined invitees, a small number of interested and qualified guests will be admitted both as audience and speakers. Financial support will be provided for all invited speakers from outside Japan. Those who wish to present papers are encouraged to submit an abstract to the organizer. Papers most appropriate to the subject will be given priority.

The majority of the presentations will be on the pharmacodynamics of drugs affecting the central nervous system, including drug metabolism, pharmacokinetics, end organ sensitivity changes during aging, and neural and humoral control of the aging liver. Papers covering the aging of only a single organ (liver or brain) may be included but the number of such papers will be limited.

Those interested in the symposium are kindly requested to contact the organizer.

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THIRD TOKYO SYMPOSIUM *LIVER AND AGING* and *LIVER AND BRAIN*

Place: Hotel Pacific, Takanawa, Tokyo, JAPAN

Date: August 20–22, 1986

Abstract Deadline: February 28, 1986

Organizer: K. Kitani, M.D.
First Laboratory of Clinical Physiology
Tokyo Metropolitan Institute of Gerontology
35-2, Sakaecho, Itabashi-ku
Tokyo, JAPAN (Telephone 03-964-1131, Ext. 3091)

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ANNOUNCEMENT

Caenorhabditis Genetics Center

GENETIC STOCKS of *Caenorhabditis elegans* are available for use by investigators initiating or continuing research on this nematode. The Center's collection of more than 1000 strains includes at least one allele of each identified gene, all available chromosome rearrangements and selected multiple mutant stocks useful for genetic mapping.

The functions of the Center include coordination of genetic nomenclature, maintenance and distribution of a bibliography of research publications, and annual revision and distribution of the *C. elegans* genetic map (see Swanson, M.M.; Edgley, M.L.; Riddle, D.L. (1984). *Caenorhabditis elegans* in *Genetic Maps 3*, O'Brien, S.J., ed. New York: Cold Spring Harbor Press, pp. 286–299). The Center acts as the central clearinghouse for naming strains, alleles, and genes. A computerized database stores the bibliographic information and the data used to generate the genetic map, as well as descriptive data on strains. The Center also distributes a *C. elegans* research newsletter by subscription.

The goal of the Center is to promote accumulation and documentation of the genetic wealth of *C. elegans* for all types of biomedical research on this organism. Established in 1979 under sponsorship of the National Institute on Aging, it is the only central repository for *C. elegans* strains. Requests for strains should include a brief statement of the research and/or research training activity for which the stocks are intended. Recipients affiliated with non-profit organizations are asked to pay postage costs, to subsequently acknowledge the Center in publications resulting from use of the strains, and to provide the Center with reprints of those publications. Inquiries should be addressed to:

M.L. Edgley, Curator or
D.L. Riddle, Director
Caenorhabditis Genetics Center
Division of Biological Sciences
Tucker Hall
University of Missouri
Columbia, Missouri 65211

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ANNOUNCEMENT

The International Association of Gerontology Announces the Third Sandoz Prize for Gerontological Research

THIS PRIZE, worth 20,000 Swiss Francs, is sponsored by SANDOZ LTD, Basel, Switzerland. Its purpose is to encourage research in all areas of gerontology and geriatric medicine including biological, medical, psychological, social, and other relevant aspects with special emphasis on multidisciplinary research programs.

The Jury consists of the Executive Committee of the International Association of Gerontology, its official Sandoz Prize coordinator, and one representative of Sandoz Ltd. The prize will be awarded on the occasion of the 1st European Congress of the International Association of Gerontology to be held in Brighton, United Kingdom, September 1987.

Applications in English should comprise a summary of the research work in 3–5 pages. Include a curriculum vitae, bibliography, and reprints of not more than three pivotal publications in English, or with extended summaries in English.

Individuals, research teams, or institutions are invited to submit their applications not later than 31 October 1986 to Prof. M. Bergener, M.D., International Association of Gerontology, Rheinische Landesklinik, Wilhelm-Griesinger-Strasse 23, D-5000 Köln 91, Federal Republic of Germany.

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ANNOUNCEMENT

NATO CONFERENCE
on
MODIFICATION OF CELL TO CELL SIGNALS DURING NORMAL AND PATHOLOGICAL AGING

Limone sul Garda, Italy, September 22–27, 1986

The aging process involves changes in neurotransmission at different levels. In general, a loss of adaptive response to environmental stimuli is observed but the experimental evidence is descriptive and scattered. The goal of this meeting is to define the "state of the art" of the field and directions for future research on the aging brain.

The following topics will be considered:

- Comparison of anatomical and neurochemical changes during normal and pathological aging
- Responses of the aging brain to drug treatments or environmental stress
- The neuro-immune and neuro-endocrine environment during aging
- Definition of therapeutic approaches (if needed) in normal aging (drugs versus life style).

For further information contact:

Dr. S. Govoni
Institute of Pharmacology and Pharmacognosy
University of Milan
Via A. Del Sarto 21
20129 Milan
Italy
Telephone: (0039)-2-741969

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ANNOUNCEMENT

WITH THIS issue of *Experimental Gerontology* we continue a new feature that we hope our readers will find useful. A Bibliographic Index will appear as space allows. It will include citations to broad areas of the current biological literature that are of potential interest to gerontologists.

The index is not intended to cover the entire biogerontological literature. Our goal is to provide current literature citations to areas of biological research that underpin biogerontology. Because gerontological research draws on so many disciplines, the Publishers hope that this new service will assist our readers in their efforts to remain current with that part of the literature from which their research in gerontology is derivative.

Our Bibliographic Index is drawn from a database produced by Pergamon Press and from which their series of "Current Advances in the Biological Sciences" is produced. The bibliographic citations of current published research that will be of interest to biogerontologists will be listed in *Experimental Gerontology* by subject category. These will include full article title, complete journal reference, authors' names, and reprint request addresses.

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Our bibliographic index will cover the following disciplines:

*NEUROSCIENCE

*CELL and DEVELOPMENTAL BIOLOGY

*BIOCHEMISTRY

*IMMUNOLOGY

*PHARMACOLOGY and TOXICOLOGY

*PHYSIOLOGY

*PLANT SENESCENCE

*GENETICS and MOLECULAR BIOLOGY

We hope you find the Bibliographic Index of interest. We invite your comments.

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REVIEW ARTICLE

EXPRESSION OF INTRACELLULAR BIOCHEMICAL DEFECTS OF LYMPHOCYTES IN AGING: PROPOSAL OF A GENERAL AGING MECHANISM WHICH IS NOT CELL-SPECIFIC

TRYGVE O. TOLLEFSBOL* and HARVEY JAY COHEN

Geriatric Research, Education and Clinical Center, Veterans Administration Medical Center, Durham, North Carolina 27705; and Center for the Study of Aging and Human Development, Duke University Medical Center, Durham, North Carolina 27710; and Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710

Key Words: immunosenescence, lymphocytes, DNA synthesis, proteins, enzymes, nucleotide effectors

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INTRODUCTION

AGING IS generally considered to be characterized by a decreased ability to adapt to various forms of stimuli and a reduction in the ability of the aging organism to maintain the homeostatic equilibrium necessary to sustain life. The age-related decrease in homeostasis is related to a progressive increase in physiologic decrements that contribute to the senescence of the aging organism. One of the most widely documented and pervasive physiologic decrements of the aged, for example, is immunosenescence. The immune system undergoes marked age-related changes which have been linked by some to several of the significant diseases associated with aging such as increased susceptibility to infection, autoimmunity disorders, cancer, vascular disease, immune-complex diseases, monoclonal gammopathies, and amyloidosis (Walford, 1974; Mathews *et al*, 1974; Hijmans and Hollander, 1977; Cohen, 1985; Zoler, 1983). The decline in immune function with increasing age has been thought by some to play an important role in the aging process (e.g., Weksler and Hutteroth, 1974; Kay, 1979; Walford, 1974). There is a progressive age-related decline in the graft vs. host reaction (Walters and Claman, 1975), delayed hypersensitivity skin reactions (Girard *et al.*, 1977), mixed lymphocyte reactions (Kishimoto *et al.*, 1978), and the response to mitogens (Weksler and Hutteroth, 1974). Although age-related changes occur in various aspects of the immune system, the most pronounced effect of aging occurs in cell-mediated immunity (Kay, 1979). The primary mediators of this aspect of the immune response are the T-cells which show a marked decline with increasing age in response to various mito-

*Address correspondence and reprint requests to: Dr. Trygve Tollefsbol, Geriatric Research, Education and Clinical Center (182), Veterans Administration Medical Center, Durham, NC 27705.
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gens such as phytohemagglutinin (PHA) and concanavalin A (ConA) (Weksler and Hutteroth, 1974).

The response of immune cells to mitogens (i.e., mitogenesis) is frequently used to assess immunosenescence and serves as an excellent model to study aging for several reasons (Makinodan, 1977). First, aging in general is characterized by a decrease in the ability to adapt to stimuli of various forms and mitogenesis affords a system whereby these cells are forced to respond to mitogenic stimulation. Second, the impaired mitogenesis of the aged appears to relate not only to cellular senescence and various age-associated diseases, but also to distinct basic biochemical defects that are found in these cells in the aged. And third, this system provides a well-controlled in vitro model which mimics the in vivo antigenic response and is amenable to precise cellular and molecular analyses. A prime focus of aging research has therefore been to characterize the impaired mitogenesis with age which could help elucidate cellular aging and the decreased immune function as an expression of cellular aging. Other factors which may be linked to the capacity for mitogenesis such as macrophage processing appear for the most part to be relatively unchanged with age (e.g., Kay, 1979). Thus, numerous investigations into the decreased immune function of the aged have focused on the impaired mitogenesis although the exact detailed mechanism(s) responsible for this impairment have not yet been fully elucidated.

A number of recent studies have indicated that the decline in mitogenesis in the aged may be due to intrinsic, biochemical, age-related defects that become apparent when these cells are subjected to the high rates of metabolism that occur during cell transformation. These studies have rapidly expanded in recent years and arose in part because the decline in mitogenesis in the aged cannot be solely attributed to age-related differences in various other factors. For instance, there is no significant change with increasing age in the total number of immune cells (Weksler and Hutteroth, 1974; Price and Makinodan, 1972; Tollefsbol and Cohen, 1984, 1985a,b) or the viability of these cells (Hung *et al.*, 1975a,b; Tollefsbol *et al.*, 1981; Tollefsbol and Cohen, 1984, 1985a,b). Likewise, there is no significant age-related change in the mitogen dose response (Ling, 1968; Verity *et al.*, 1983) or the binding, affinity, and number of mitogen receptors (Heidrich, 1973; Hung *et al.*, 1975a; Weksler, 1983a). The number of responding cells has been reported to be decreased with age (Weksler, 1983b) although this has been contested (Verity *et al.*, 1983).

Other possible cellular explanations for the impaired mitogenesis of the aged have also been investigated. The studies of the effect of age on shifts in the subpopulations of immune cells have not been entirely conclusive. The proportions of B and T cell subsets have been reported to be unchanged with age (Callard and Basten, 1977; Weksler, 1983b; Globerson *et al.*, 1981; Deviere *et al.*, 1985) although some studies have indicated that certain subsets such as suppressor or helper cells may change with age (Yunis and Lane, 1979; Weksler, 1983a; Talal, 1977; Segre and Segre, 1976). Kennes *et al.* (1981) have indicated that the changes if any of the subpopulations may be too slight to account for the markedly impaired immunological functions. The decline in cellular lymphokine secretion likewise may not be the result of suppression by suppressor cells (Thoman and Weigle, 1981), a decrease in helper cells (Chang *et al.*, 1982), or changes in absorption by responding cells (Chang *et al.*, 1982). Moreover, lymphocyte proliferation is impaired with increasing age when assessed through an interleukin-independent pathway (Wu *et al.*, 1985) and supplementation of cultures of lymphocytes from aged subjects has been reported to fail to restore (Gillis *et al.*, 1981; Kennes *et al.*, 1983), partially restore (Chang *et al.*, 1982), or

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fully restore (Thoman, 1985) the lymphocyte response to the level of the young. Serum factors transferred between young and aged subjects scarcely modify mitogenesis (Price and Makinodan, 1972) and transfer of lymphocytes between young, old, and thymectomized subjects indicate that the age-related defect may be intrinsic to immune cells (Weksler, 1983b). In fact, several other studies have indicated that the decline with age in mitogenesis is due to some type of intrinsic defect in these cells (Price and Makinodan, 1972; Heidrick, 1973; Makinodan *et al.*, 1977). Price and Makinodan (1972) reported that 10% of the normal age-related decline in immune function can be attributed to changes in the cellular milieu while about 90% of the decline can be attributed to changes intrinsic to the cells.

The concept that immunosenescence is due at least in part to mechanisms encoded within the cell is gaining increasing experimental support and is not inconsistent with the direction of research in other aging systems. The purpose of this work is to analyze the findings of age-related changes in the intracellular chemistry and metabolism of lymphocytes in the aged and to link individual findings that may collectively play important interdependent roles in the impaired mitogenesis of the aged. Although the focus of this work is on the basic chemistry of the lymphocyte with increasing age, every attempt is made to relate these findings to similar defects which occur in various other cell types with the intention that many of these basic defects may not be entirely cell-specific but may, in fact, be generally applicable and related to common basic mechanisms of aging. A potential general underlying mechanism of cellular aging is proposed from this analysis based on a genetic "slowing-cycle" effect of transcription, translation, and enzyme induction with the age-related impaired mitogenesis and immunosenescence presented as example of the expression of this basic mechanism.

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NUCLEIC ACIDS AND TRANSCRIPTION

It is well established that mitogen exposure stimulates DNA synthesis in lymphocytes (e.g., Dukor and Dietrich, 1967; Rodey and Good, 1969; Greaves and Roitt, 1968) which is usually assessed by the incorporation of radiolabeled thymidine into the transforming cells. For example, Fig. 1 shows that DNA synthesis is significantly induced in mitogen-exposed cells by at least 24–48 h of culture during the same interval that the percent of transformed cells increases from a basal level of about 10 up to 60% (Tollefsbol and Cohen, 1985c). The increased incorporation of thymidine into the proliferating cells is not simply due to an increase in the number of cells since the thymidine incorporation is significantly increased per viable cell (Fig. 1). The blastogenic index, which is the ratio of thymidine incorporation of stimulated to unstimulated cells (i.e., control cells), also is significantly increased by 24–48 h of culture but markedly declines after most of the cells have transformed (Tollefsbol and Cohen, 1985c).

The incorporation of thymidine into mitogen-stimulated lymphocytes from aged humans and animals is generally believed to be markedly impaired (e.g., Hori *et al.*, 1973; Weksler and Hutteroth, 1974) although there have been some conflicting results with different mitogens. For instance, Cylonkowska and Korlak (1979) reported an age-related decrease of thymidine incorporation into PHA-stimulated lymphocytes but not for cells exposed to Con A although most other studies have indicated that DNA synthesis is also impaired in Con A-stimulated lymphocytes with increasing age (e.g., Kruisbeek, 1976; Cheung *et al.*, 1983; Weksler, 1983a). Nagel *et al.* (1982) reported decreased thymidine in-

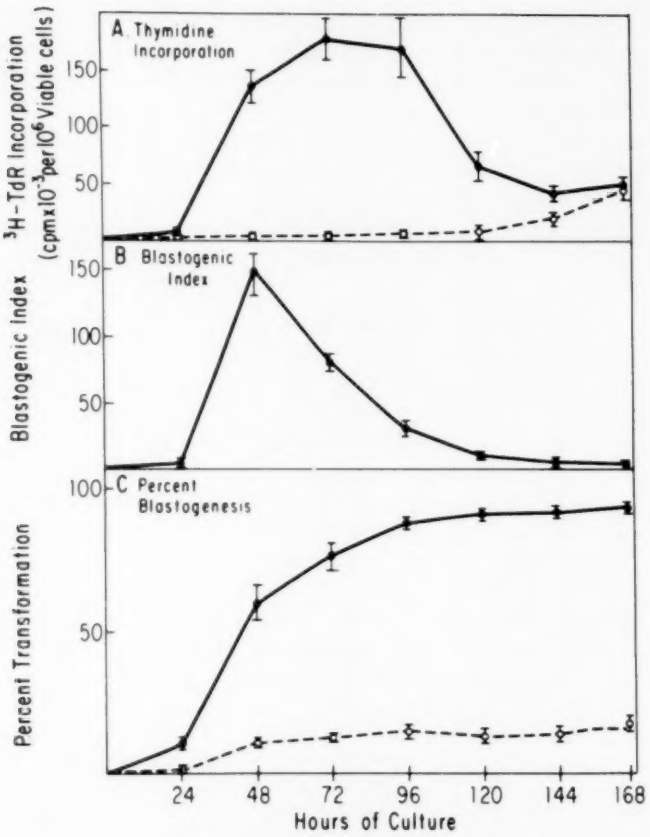

FIG. 1. Thymidine incorporation and blastogenesis of PHA-stimulated lymphocytes. The incorporation of tritiated thymidine (^{3}H-TdR) and the percent transformation was assessed in PHA-stimulated and control cultures from ten subjects at 24-hour intervals over 7 days of culture. (A) Thymidine incorporation: Stimulated (10 μg PHA/ml) and nonstimulated (control) cells were pulsed with ^{3}H-TdR and the absolute incorporation of ^{3}H-TdR was determined per viable cell. Each value represents the mean ± SEM of duplicate determinations from the ten subjects. Solid line, stimulated cells; dashed line, nonstimulated cells. (B) Blastogenic index: The blastogenic index was determined by dividing the duplicate ^{3}H cpm in the stimulated viable cells by the duplicate ^{3}H cpm in the hour-matched nonstimulated viable cells for each of the ten subjects: Blastogenic index = ^{3}H cpm/10^6 stimulated viable cells/^{3}H cpm/10^6 nonstimulated viable cells. Values represent the mean ± SEM. (C) Percent blastogenesis: Cellular morphology was assessed on Trypan blue-stained cells for transformation. The percent transformation was assessed in quadruplicate for each of the ten subjects at each indicated hour of incubation. Values represent mean ± SEM. Solid line, stimulated cells; dashed line, nonstimulated cells.

corporation with increasing age in response to Con A, PHA, phorbol-acetate, but not pokeweed mitogen (PWM) although other studies have reported an age-related decrease in thymidine incorporation with PWM (e.g., Mathies *et al.*, 1973; Tollefsbol and Cohen 1984). The B-cell mitogen, lipopolysaccharide (LPS), also elicits an impaired thymidine incorporation in the aged (Abraham *et al.*, 1977; Cheung *et al.*, 1981) but the decreased

DNA synthesis is more apparent with T-cell-specific mitogens than B-cell-specific mitogens (Weksler, 1983a). However, even the T-cell studies, as exemplified with the use of PHA or Con A as the mitogen, have not gone without occasional conflicting reports. For instance, Portaro *et al.* (1978) found no age-related decrease in DNA synthesis in PHA-stimulated lymphocytes and indicated that their subjects represented a more restricted population of healthy elderly subjects although Goodwin *et al.* (1982) studied 300 healthy elderly subjects and reported a substantial impairment in thymidine incorporation with PHA in the aged. Several other studies reporting an age-related decrease in DNA synthesis in response to PHA also selected only healthy subjects who had been screened for any drugs or disorders which may affect the fidelity of lymphocyte culturing (e.g., Tollefsbol and Cohen, 1985b). A group of chronically ill subjects was assessed for age-related changes in DNA synthesis (Goodwin *et al.*, 1982) and the decreased response was similar to that seen in healthy subjects indicating that age and not accompanying illness was the cause for the impaired thymidine incorporation of these cells.

The kinetics of the age-related decline in DNA synthesis in PHA-stimulated lymphocytes has been demonstrated both in vivo and in vitro. Hori *et al.* (1973), using PHA-stimulated mouse spleen lymphocytes, found a decreasing thymidine incorporation with progressive aging of the animals. The decline in DNA synthesis of T cells was apparent by adulthood and continued to decline with increasing age so that by old age the DNA synthetic rate of the cells was much less than that of the young. The kinetics of the decline in thymidine incorporation with age has also been assessed in vitro. Fig. 2 shows that with PHA-stimulation the thymidine incorporation per viable cell is significantly less and delayed in the aged as compared to the young and that this significant impairment continues through 72 h of culture after which time the thymidine incorporation is less in both age groups and is not significant (Tollefsbol and Cohen, 1985b). Assessment of the thymidine incorporation and blastogenic index reveals that there is a significant age-related difference in DNA synthesis by at least 24–48 h of culture (Fig. 2) at which time these cells also begin to show a significant age-related decrease in the number of transformed cells. Many other studies have also shown a decrease in DNA synthesis by PHA-stimulated lymphocytes in the aged (e.g., Pisciotta *et al.*, 1967; Hallgren *et al.*, 1973; Delespesse *et al.*, 1974) which is not related to mitogen receptor response (Weksler, 1983b) or mitogen dose response (Verity *et al.*, 1983; Kennes *et al.*, 1981). Likewise, this decrease is not due to an age-related impairment in an extractable cytoplasmic protein previously identified to in part help induce DNA synthesis during mitogenesis (Gutowski *et al.*, 1984).

Verity *et al.* (1983) assessed DNA synthesis in the young and aged for PHA-stimulated human lymphocytes using a continuous labeling method that leaves the cultures undisturbed until the time of the assay. They also found that DNA synthesis is significantly impaired and delayed in the aged through use of this method. Since exposure to the radiolabel may cause chromosomal damage to which the aged may be more sensitive, Staiano-Coico *et al.* (1983) assessed the proliferative response of lymphocytes with flow cytometry and the incorporation of tritiated thymidine in the young and aged. The proliferative response of the cells was decreased with age as was the DNA synthetic rate although the cells from the aged were apparently more sensitive to the radiolabel. However, this effect was not pronounced enough to account for the markedly impaired DNA synthetic rates with age.

Thus it is apparent that there is an age-related decrease in DNA-synthetic rates in lym-

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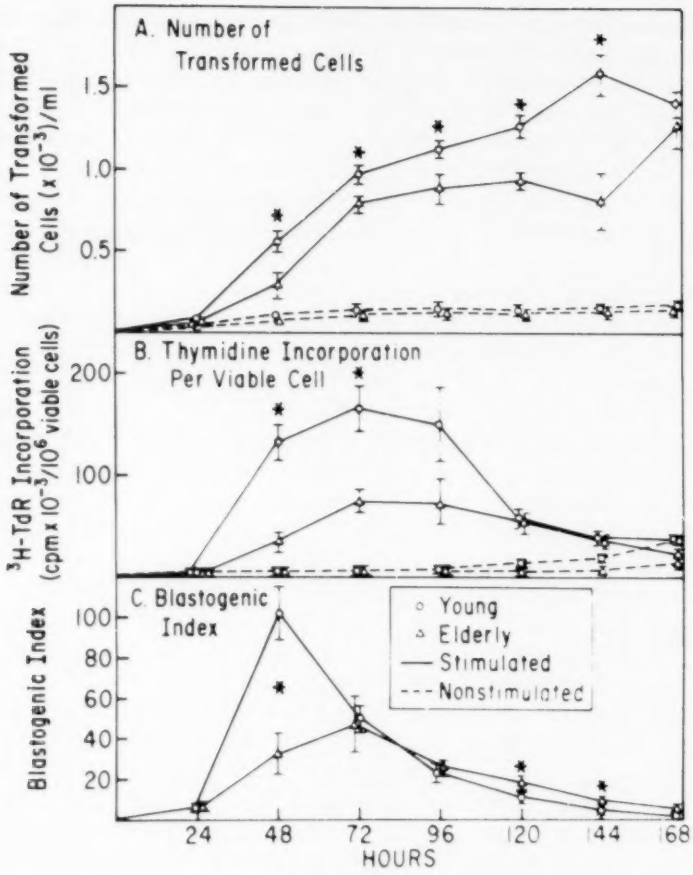

FIG. 2. Kinetics of transformation of stimulated lymphocytes with age in extended cultures. All depicted values represent the mean ± SEM from 14 young and 14 elderly subjects and significantly different values ($P \leq 0.05$) with age are indicated with an asterisk. Solid lines represent PHA-stimulated cultures and dashed lines represent cultures which contained no mitogen. The values for the young are indicated with circles and the values for the elderly are indicated with triangles. A) Number of transformed cells. Cellular morphology was assessed on Trypan Blue-stained cells for percent transformed cells in quadruplicate. Transformed cells were identified by a high nuclear to cytoplasmic ratio and large cell size as compared to small resting lymphocytes. The percent transformed cells was then multiplied by the number of cells in each culture (assessed in quadruplicate by Coulter counting) to obtain the number of transformed cells. B) Thymidine incorporation. The cells were pulsed in duplicate for 6 h with 1.25 μCi ^{3}H-TdR/10^6 cells/ml at each indicated hour of incubation except freshly isolated cells (zero h). Each value represents the ^{3}H thymidine cpm incorporated per 10^6 stimulated or nonstimulated viable cells. C) Blastogenic index. The blastogenic index is the ratio of the ^{3}H thymidine cpm incoporated per 10^6 stimulated viable cells to the ^{3}H thymidine cpm incorporated per 10^6 nonstimulated viable cells for the same pulse period.

phocytes, which is most pronounced in T-cells undergoing mitogenesis. Kinetic studies show that this decrease occurs with increasing age of the donor in vivo and the rate of DNA synthesis is also impaired and delayed in vitro. The exact cause of this impairment in DNA synthesis in the aged and the role that it plays in the impaired mitogenesis of the aged has not yet been fully elucidated.

PROTEINS AND TRANSLATION

An important early mitogenic event is the initiation of protein synthesis which occurs by 2–3 h after mitogen addition and is accompanied by a linear increase in the percentage of active ribosomes (Ahern and Kay, 1975; Jagus and Kay, 1979; Cooper and Baverman, 1977). This early increase in protein synthesis with mitogenesis precedes the onset of DNA synthetic induction (Ahern and Kay, 1975) and upon mitogenic stimulation an increase in RNA content precedes the increase in DNA content (Darzynkiewicz *et al.*, 1976). The marked increase in polysomes upon mitogenic exposure of lymphocytes (Cooper and Braverman, 1977) occurs in conjunction with the production of a variety of specialized proteins such as lymphokines whose biological activities subserve the immune reaction (Lawrence and Landy, 1969; Cooper and Braverman, 1977). Since interleukin 2 (IL-2), a lymphokine which plays a role in lymphocyte proliferation, is decreased with age (Gillis *et al.*, 1981; Thoman and Weigle, 1981), Cheung *et al.* (1983) examined the protein synthetic activity of Con A-stimulated lymphocytes from the laboratory rat and determined that with increasing age of the rat the stimulated lymphocytes were less able to increase protein synthesis indicating that an age-related defect in protein synthesis may relate to the decreased IL-2 production and mitogenesis in these animals. However, IL-2 supplementation may not restore or only partially restore immune function in the aged (Gillis et al, 1981; Kennes *et al.*, 1983; Chang *et al.*, 1982) and the induction of lymphocyte activation is decreased in the aged when assessed in systems which are independent of this lymphokine (Wu *et al.*, 1985). In fact, Wu *et al.* (1985) have indicated that the decline in IL-2 production with increasing age does not appear to be the main cause for the age-related decline in lectin-induced lymphocyte proliferation. Tollefsbol and Cohen (1985a), in a study of a population of healthy young and elderly humans, assessed the kinetics of protein synthesis over 72 h of culture in PHA-stimulated lymphocytes and discovered that protein synthesis was markedly impaired in the aged (Fig. 3) and this impairment preceded and then paralleled the kinetics of the age-related impairment in DNA synthesis and mitogenesis (Tollefsbol and Cohen, 1985a,b). By 72 h of culture the incorporation of radiolabeled leucine in stimulated cells from elderly persons is about half that for the young. This impairment in protein synthesis could relate to the cause for the impaired induction of numerous enzymes which are requisite for mitogenesis or to numerous other factors involved in cell function which are dependent upon protein synthesis (Tollefsbol and Cohen, 1985a).

It has been known that protein synthesis in fibroblasts is required throughout the G1 interval for the initiation of DNA synthesis (e.g., Terasima and Yasukawa, 1966; Wiebel and Baserga, 1969; Schneiderman *et al.*, 1971) and that protein synthesis inhibitors prevent the initiation of DNA synthesis (Terasima and Yasukawa, 1966; Schneiderman *et al.*, 1971). Since the cause of the age-related decrease in thymidine incorporation in PHA-stimulated lymphocytes was not known and these cells have a decreased protein synthetic

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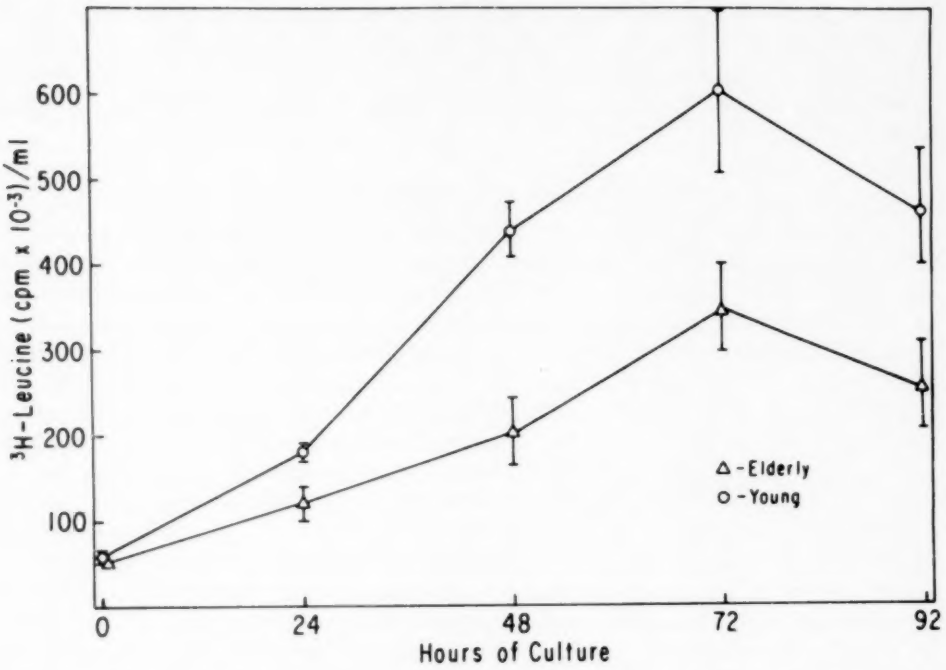

FIG. 3. Leucine incorporation by phytohemagglutinin-stimulated lymphocytes. After culture, for the indicated intervals, cells were washed and adjusted to 1×10^6 cells/ml in leucine free MEM and the incorporation of [^{3}H] leucine/ml determined in quadruplicate at 0 and 6 h of incubation. Depicted values are the differences between the incorporation at the 6 h and 0 h determinations. All values are the mean ± S.E.M. from 10 young subjects (mean age = 28 years; age range = 23–32 years) and 10 elderly subjects (mean age = 75 years; age range = 70–80 years). The values for the young are indicated with circles and the values for the elderly are indicated with triangles.

rate (Tollefsbol and Cohen, 1985a), cycloheximide, a protein synthesis inhibitor, was employed to determine in part if the impaired thymidine incorporation in the aged during mitogenesis could be related to the finding of the age-related decrease in protein synthesis (Tollefsbol and Cohen, 1985a). Cycloheximide indeed inhibited thymidine incorporation in PHA-stimulated lymphocytes indicating that a decreased synthesis of enzymes which are essential for DNA synthesis such as the DNA polymerases, thymidine kinase, etc. could result in the impaired thymidine incorporation in mitogen-stimulated lymphocytes in the aged. This is consistent with the reports that the burst in protein synthesis precedes DNA synthesis in young subjects' PHA-stimulated lymphocytes (Ahern and Kay, 1975) and provides a logical explanation for the decreased DNA synthesis during mitogenesis in the aged. Other age-related aspects of mitogenesis such as rate of entry into *S* phase and reduced lymphokine secretion may also be explained by the impaired protein synthesis in PHA-stimulated lymphocytes. Certainly, an age-related defect in a process as basic as protein synthesis could be envisioned to affect many cellular functions, effectors, or biochemical pathways which may collectively play a critical role in impaired mitogenesis (Tollefsbol and Cohen, 1985a) and cellular senescence of the aged.

PROTEIN DEGRADATION AND ALTERED ENZYMES

The cause for the age-related decrease in protein synthesis in transforming lymphocytes is not known although it could be due to several different factors including a progressive age-related decrease in the genetic expression of protein synthesis, interference of protein synthesis through an accumulation of altered proteins, or decreased protein degradation causing an impaired recycling of amino acids for protein synthesis. Certainly genetic influences play an important role in aging as exemplified by the interspecies variability and intraspecies constancy of longevity and most of the expression of the genetic apparatus is through the synthesis of proteins which have widespread essential functions requisite to the viability of the organism. Since the decreased protein synthesis of aging organisms appears to be universal (Richardson, 1980; Tollefsbol and Cohen, 1986) and may be under genetic influence, a cause for the decreased protein synthesis of mitogenesis in the aged could be due to a genetically programmed decreasing expression of this essential function of the cells.

The decreased protein synthesis of PHA-stimulated lymphocytes in the aged may also be due to defects in protein degradation (Tollefsbol and Cohen, 1985a). It is known that there is a slowing of protein degradation with increasing age that could cause an accumulation of altered protein forms in cells from the aged (e.g., Rothstein, 1975; Tollefsbol and Cohen, 1986). In support of this, PHA-stimulated human lymphocytes have an age-related increase in protein content in the face of a decrease in protein synthesis indicating that protein degradation may be defective in these cells and could cause an accumulation of altered protein forms (Tollefsbol and Cohen, 1985a). In fact, an accumulation with mitogenesis of a more labile altered enzyme form related to the normal degradation of this enzyme has been found in the aged (Tollefsbol and Cohen, 1984). As part of its normal catabolic process in the young, this enzyme, triosephosphate isomerase, undergoes sequential spontaneous deamidations which renders the enzyme altered and more susceptible to protein degradation (Yuan *et al.*, 1981). This altered enzyme, which is more labile to thermal denaturation (Tollefsbol *et al.*, 1982), accumulates during aging and mitogenesis (Tollefsbol and Gracy, 1983; Tollefsbol and Cohen, 1984). This accumulation of altered forms of proteins during mitogenesis in the aged may increase the molecular entropy of the cell and interfere with requisite inductive mechanisms such as protein synthesis (Tollefsbol and Cohen, 1985a).

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ION AND COFACTOR ALTERATIONS

Certain ions which appear to play a role in mitogenesis through early biochemical transport events or as cofactors of enzyme reactions have been assessed. Since calcium ions influx into transforming lymphocytes shortly after stimulation, Kennes *et al.* (1981a) examined the transport of calcium during mitogenesis in young and aged subjects. No age-related changes in the enhancement of calcium transport during mitogenesis were found. However, PHA-stimulated lymphocytes from the aged were found to be more sensitive to calcium chelating agents indicating that T-cells of aged subjects are more sensitive to calcium loss (Kennes *et al.*, 1981b) which may relate to the role of calcium as a cofactor in several enzyme reactions. Moreover, blocking the calcium channel inhibits mitogenesis to a greater degree in aged as compared to young subjects. Wu *et al.* (1985) have also reported a decreased T-cell responsiveness with age to calcium ions and suggested that calcium ions may play a role in the age-related decline in mitogenesis. Zinc is also impor-

tant cofactor in many enzyme reactions although its addition to PHA-stimulated lymphocytes appears to cause a suppression of thymidine incorporation in the aged (Rao *et al.*, 1979). Thus, the exact role that these cofactor changes may play in mitogenesis in the aged remains uncertain.

The effect of age on Na^+, K^+-ATPase induction in mitogenesis has also been assessed (Kennes *et al.*, 1982). Like changes in calcium influx, changes in membrane permeability to K^+ and activation of the membrane associated Na^+, K^+-ATPase has been reported to play a role in mitogenesis (Hume and Weidermann, 1980) although no age-related differences in the magnitude and timing of the PHA-induced increase in K^+ influx were found (Kennes *et al.*, 1982). Moreover, studies with digoxin indicated that the triggering mechanisms of membrane-associated Na^+, K^+-ATPase induced by PHA occurs adequately in stimulated lymphocytes from aged subjects. These studies on ion-system changes during mitogenesis in the aged indicate that ions are able to influx into the cell adequately but that stimulated cells from the aged may be more sensitive to decreases in certain ions such as calcium which serves as a cofactor in numerous enzyme reactions.

ABERRATIONS OF NUCLEOTIDE EFFECTORS

Variations in cyclic nucleotides are also early events involved in mitogenesis and may play a role in the regulation of mitogenesis (Parker *et al.*, 1974). Some studies have indicated that cAMP is a positive effector of mitogenesis (Parker *et al.*, 1974; Wedner and Parker, 1976; Parker, 1979) while others have indicated a negative role for cAMP but a positive role for cGMP in mitogenesis (Hadden *et al.*, 1972; Coffey *et al.*, 1977). Watson (1975) has suggested that the ratio of cAMP/cGMP may be an important regulator of mitogenesis. Tam and Walford (1978, 1980) reported an age-related decrease in the levels of cAMP but an increase in cGMP levels with a consequent decrease in the cAMP/cGMP ratio in resting lymphocytes. Kinetic patterns of responses of the cyclic nucleotides were changed with increasing age in response to PHA, Con A, and PWM (Tam and Walford, 1978). Mark and Weksler (1982) reported no change with increasing age in the cAMP and cGMP levels in resting and Con-A stimulated lymphocytes while Fulop *et al.* (1984) have reported age-related changes in cyclic nucleotide levels consistent with the changes reported by Tam and Walford (1978, 1980) in resting and phagocytosing polymorphonuclear leukocytes.

The levels of ATP and ADP and the energy status (ATP/ADP) also undergo changes during mitogenesis. The ATP content of PHA-stimulated lymphocytes markedly increases by at least 24–48 h of culture in the young and remains elevated (Chapman *et al.* 1981; Verity *et al.*, 1983). Chapman *et al.* (1981) reported an age-related decrease in energy status of control unstimulated cells whereas Verity *et al.* (1983) found no difference with age in the adenine content of unstimulated lymphocytes. A decrease in the energy status (ATP/ADP) occurs during mitogenesis in the aged due to a decrease in ATP levels (Chapman *et al.*, 1981) and the normal increase in ATP production in the young is also significantly delayed with age in response to PHA (Verity *et al.*, 1983). The cause for the impairment and delay in ATP production during mitogenesis in the aged was suggested by Verity *et al.* (1983) to be related to a mitochondrial dysfunction, although the TCA cycle and respiration are not significantly changed during mitogenesis (McKeehan, 1982; Tollefsbol and Cohen, 1985c; Kester *et al.*, 1977; Polgar et al, 1968; Hume and Weidemann, 1979). However, glycolysis undergoes a marked induction during

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mitogenesis (Kester *et al.*, 1977; Rogers *et al.*, 1980; Tollefsbol and Cohen, 1985a–c) and produces high levels of ATP (Polgar *et al.*, 1968) and therefore would be expected to markedly affect the adenylate charge of these cells and relate to the impairment and delay in ATP production during mitogenesis in the aged (Tollefsbol and Cohen, 1985b). In fact, the normal induction of glycolysis is significantly impaired and delayed during mitogenesis in the aged (Tollefsbol *et al.*, 1981; Tollefsbol and Cohen, 1985b) and parallels the impairment and delay in ATP production in these cells (Chapman *et al.*, 1981; Verity *et al.*, 1983; Tollefsbol and Cohen, 1985b).

Two important factors involved in the increased metabolic activity associated with cell growth are the adenine and pyridine nucleotide levels (Barnes *et al.*, 1972; Morton, 1958). Besides a marked increase in ATP production during mitogenesis in the young, PHA stimulation also induces a marked increase in NAD(H) levels (Blomquist *et al.*, 1976; Chapman *et al.*, 1981, 1983) and stimulated cells from the aged have an impaired increase in NAD(H) levels (Chapman *et al.*, 1981, 1983). There is a significant age-related difference in NAD^+ levels during mitogenesis (Chapman *et al.*, 1981, 1983) while the age-related changes in NADH levels may (Chapman *et al.*, 1981) or may not (Chapman *et al.*, 1983) be significant. The redox state (NAD^+/NADH) decreases in the young during mitogenesis but does not significantly change with mitogenesis in the aged (Chapman *et al.*, 1981). There is no change in NADPH levels in the young during mitogenesis but with increasing age the NADPH levels and the total NADP(H) decreases (Chapman *et al.*, 1981). No age-related change in NAD(H) or NADP(H) levels occurs in unstimulated cells (Chapman *et al.*, 1981). The marked increases in redox potential during mitogenesis in the young and the decrease in this potential in the aged may be related in part to the increased glycolytic flux and the impaired induction of this pathway during mitogenesis in the aged (Tollefsbol and Cohen, 1985b,c; Chapman *et al.*, 1981).

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ENZYMOLOGY

Protein/enzyme metabolism is one of the most crucial aspects of cell function and may be critical to the intracellular cause of impaired mitogenesis of the aged. Enzymes catalyze diverse chemical reactions of the cell and are especially important in cells which are undergoing an adaptive response such as mitogenesis. A molecular defect or impaired catalytic activation of just one key enzyme may interfere with the flux of several biochemical pathways and consequently impair the cellular response. For instance, inhibition of protein synthesis also inhibits DNA synthesis and mitogenesis. This might be due to the inhibition of the requisite induction of enzymes such as DNA polymerase which are necessary for DNA synthesis (Tollefsbol and Cohen, 1985a). Thus, key enzymes involved in DNA synthesis may be impaired in induction with age and in part relate to the impaired thymidine incorporation into these cells. Several enzymes have been assessed for age-related changes in catalytic activity in lymphocytes. For example, adenosine deamidase, an enzyme of purine metabolism, is deficient in severe, combined immunodeficiency disease which affects both B and T-cells (Giblett *et al.*, 1972; Parkman, 1975). Scholar *et al.* (1980) therefore determined the catalytic activity of adenosine deamidase in lymphocytes from subjects of different ages with the idea that a deficiency of this enzyme in the aged could be related to the impaired immunity. However, no age-related change in the catalytic activity of adenosine deamidase in resting or mitogen-stimulated lymphocytes was found although Tam and Walford (1980) reported a paradoxical elevation

of adenosine deamidase in lymphocytes with increased subject age. Purine nucleoside phosphorylase, also a purine salvage enzyme, is deficient in immunodeficiency involving severe T-cell dysfunction (Stoop *et al.*, 1977; Sandman *et al.*, 1977). In older subjects, this enzyme has been reported to be decreased in catalytic activity in lymphocytes and the decline in nucleoside phosphorylase activity may be concurrent with the age-related decline in T-cell function (Scholar *et al.*, 1980).

Other enzymes have also been reported to change in lymphocytes with increasing age of subjects. The ability of T-cells to proliferate may be related to an enhancement of the hydrolytic enzyme acid phosphatase (Mysliwska *et al.*, 1985a). With increasing subject age this enzyme is decreased in catalytic activity in resting lymphocytes (Myslwiska *et al.*, 1985b) and the age-related differences become more pronounced after stimulation with PHA. Mysliwska *et al.* (1985c) also reported that an age-related decline in acid phosphatase activity may also be related to the decline in lymphocyte cytotoxic potential of the aged. The decreased catalytic activation of this enzyme, which is in part involved in protein degradation (Mysliwska *et al.*, 1985a), could relate to a protein degradation defect during mitogenesis in the aged (Tollefsbol and Cohen, 1985a). However, other studies besides those of Mysliwska *et al.* (1985a-c) have reported an age-related increase (Sleyster and Knook, 1980), decrease (Kurnick and Kernen, 1962), or no change (Palatroni *et al.*, 1982; Wilson *et al.*, 1982) in the activity of acid phosphatase. Another enzyme that has been associated with immunonodeficiency, etco-5′-nucleotidase (Webster *et al.*, 1978), also has been reported to be decreased with subject age in human lymphocytes (Boss *et al.*, 1980) but was not assessed with mitogenesis. Heidrick *et al.* (1980) found cyclic nucleotide phosphodiesterase activity to be enhanced in splenic cells from aged as compared to young mice which could be related to the reported decrease in cAMP in these cells (Tam and Walford, 1978, 1980). Paradoxically, the levels of adenylate cyclase, the generating enzyme for cAMP, and guanylate cyclase, the generating enzyme for cGMP, are increased and decreased respectively with increasing age in human lymphocytes in the face of an age-related decrease in cAMP and an increase in cGMP (Tam and Walford, 1980). However, altered intracellular compartmental distributions of these enzymes in the lymphocytes from the aged were reported which could, in part, explain the age-related paradoxical changes in the catalytic activity of these enzymes in the opposite direction of the formation of their reaction products (Tam and Walford, 1980).

Although with increasing age, several enzymes have been shown to be altered in catalytic activity, very few studies have related such a defect to biochemical pathways which are requisite for mitogenesis. For instance, if certain biochemical pathways are necessary and basic to mitogenesis and these pathways increase in flux as part of the increased metabolism of mitogenesis, then the age-related induction of the enzymes of these pathways would be of prime interest if the flux of any of these pathways is decreased during mitogenesis in the age. Such an impairment in glycolysis has been found in transforming lymphocytes from aged subjects (Tollefsbol et al, 1981; Tollefsbol and Cohen, 1985b). Cells which are subjected to the metabolic stress of transformation (i.e., increase in the rate of intracellular metabolism) markedly increase their requirement for macromolecular synthesis and energy production which is accompanied by a high rate of glycolysis (e.g., Polgar *et al.*, 1968; Tollefsbol and Cohen, 1985c; Roos and Loos, 1970, 1973a). The increase in glycolysis is not merely an anaerobic response but is an essential function of these cells to help supply the anabolic substrates and energy for the high

rate of metabolism required for mitogenesis (Ling, 1968; McKeehan, 1982; Hume and Weidemann, 1979; Roos and Loos, 1973b). Mitogen-stimulated lymphocytes have an increased flow of glucose carbon into protein, nucleic acids, and lipid pools (Hume and Weidemann, 1979) which could explain the elevated levels of for instance, phosphoribosylpyrophosphate, glycerol, glycerol phosphate, and numerous other anabolic compounds in these cells. Moreover, changes in respiration and glycogen metabolism appear not to be a general property of transforming cells (McKeehan, 1982; Polgar *et al.*, 1968; Tollefsbol and Cohen, 1985c; Hedeskov, 1968). Glucose is the major fuel source for these cells (Ardawi and Newsholme, 1982; Englehardt and Metz, 1971) and the rate of glucose utilization and lactate production is markedly increased with mitogenesis (e.g., Hedeskow, 1968; Englehardt and Metz, 1971; Tollefsbol and Cohen, 1985c). The addition of PHA to peripheral lymphocytes induces a rapid increase in the flux of glycolysis (e.g., Hedeskow, 1968) and lymphocytes utilize almost all of their major fuel supply (i.e., glucose) via glycolysis (Ardawi and Nedvsholme, 1982, 1983; Englehardt and Metz, 1971). Moreover, blocking of glycolysis with monoiodacetate or sodium fluoride or the withdrawal of glucose as a fuel source inhibits cell transformation (Kay, 1976; Roos and Loos, 1970; Hume et al, 1978). Kester *et al.* (1977) found a coordinate increase of the glycolytic enzymes at 72 h of incubation with mitogens in transforming lymphocytes as have others (Tollefsbol *et al.*, 1981; Tollefsbol and Cohen, 1985a–c, Rogers *et al.*, 1980; Wang *et al.*, 1980). Isozyme changes also occur in the glycolytic enzymes during mitogenesis in the young which could be related to changes in the control of the pathway during its induction (Kester *et al.*, 1977; Rogers *et al.*, 1980; Hellung-Larson and Andersen, 1968; Tollefsbol and Gracy, 1980).

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In healthy young subjects the glycolytic enzymes significantly increase by at least 24–48 h of PHA-stimulation during the time frame that these cells show a significant increase in the glycolytic product, ATP, and in the percent blastogenesis (Tollefsbol and Cohen, 1985c; Verity *et al.*, 1983; Chapman *et al.*, 1981). This increase is apparent whether the increase in activity is determined per 10^6 cells, viable cell, mg protein, or transformed cell indicating that this is an intrinsic intracellular adaptive response of these cells (Tollefsbol and Cohen, 1985b,c). The maximal glycolytic enzyme activity reached during mitogenesis is about 4-fold over control cells and the catalytic activity remains elevated for as long as the cells are transformed apparently to maintain a high supply of energy and anabolic substrates for these cells (Tollefsbol and Cohen, 1985b,c; Kester *et al.*, 1977). No significant induction of enzymes from the TCA cycle and the hexosemonophosphate shunt has been found (Tollefsbol *et al.*, 1981; Tollefsbol and Cohen, 1985b,c; Kester *et al.*, 1977). Glucose utilization is significantly increased but follows the increase in glycolytic enzymes (Tollefsbol and Cohen, 1985b,c) indicating that the increase in glycolysis during mitogenesis is due to an internal inducing mechanism and is not directly due to substrate activation (Tollefsbol and Cohen, 1985b,c; Englehardt and Metz, 1971; Singh *et al.*, 1974).

Since glycolysis plays a basic and crucial role in transforming cells, Tollefsbol *et al.* (1981) examined the levels of all of the glycolytic enzymes at 72 h of culture in PHA-stimulated and unstimulated cells and found that there was a significant impairment in the ability of mitogenic cells to increase the glycolytic enzymes in the aged as compared to the young. Kinetic studies indicated that the glycolytic enzymes were significantly impaired and delayed in induction (Fig. 4) similar to the type III impairment of enzyme induction

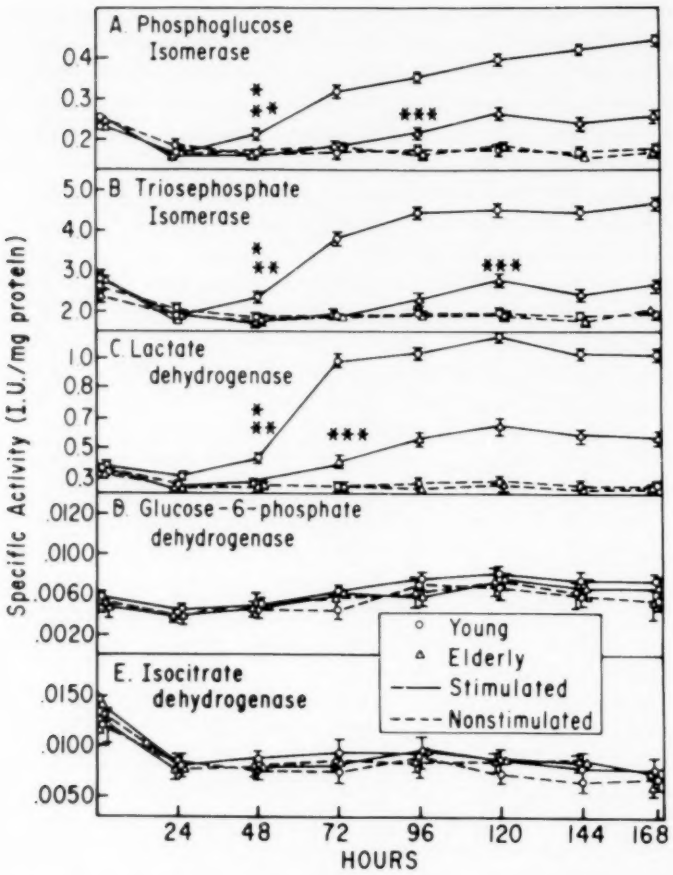

FIG. 4. Culture kinetics of glycolytic enzyme specific activity in transforming cells from aged and young subjects. Each value represents the mean ± SEM specific activity (I.U./mg) for each indicated enzyme in stimulated (solid lines) and nonstimulated (dashed lines) cultures from 14 young (circles) and 14 elderly (triangles) subjects over a 7-day culturing period. A single asterisk marks the hour of culture that a significant ($P < 0.05$) reduction in specific activity in stimulated cells from the elderly as compared to the young is first noted. Significant increases in specific activity in stimulated cultures over the control are indicated with two asterisks for the young and three asterisks for the elderly. All differences after each indicated significant value for any of the three comparisons [young stimulated vs. elderly stimulated (*), young stimulated vs. young nonstimulated (**), and elderly stimulated vs. elderly nonstimulated (***)] continued to be significant throughout the rest of the culture.

(i.e., delayed and reduced) reported by Adelman to occur in aging systems (Tollefsbol and Cohen, 1985b, 1986; Adelman, 1977). The delay and reduction in glycolytic enzyme induction during mitogenesis in the aged parallels the delay and impairment in the normal increase of the products of glycolysis such as ATP, as essential growth factors for mitogenesis, and it also parallels the age-related delay and impairment in mitogenesis (Tollefsbol and Cohen, 1985b; Verity *et al.*, 1983; Chapman *et al.*, 1981; 1983). There is a significant

age-related impairment in glucose utilization in these cells during mitogenesis, but this impairment follows the kinetics of the impairment in glycolytic enzyme induction (Tollefsbol and Cohen, 1985b). The age-altered glycolytic enzyme induction is intrinsic to the cell since this defect is apparent per viable cell, mg protein, and transformed cell (Fig. 5) and therefore cannot be attributed to changes in viability or decreased number of transformed cells in the aged (Tollefsbol and Cohen, 1985b). Lengle *et al.* (1978) reported that adult mice have less glycolytic activity in lymphocytes as compared to young mice and Dieter *et al.* (1984) found that thymocytes have decreased activity of glycolytic enzymes and loss of energy production by glucose oxidation with age. They proposed that the impaired T-cell function may be due to an age-related impairment of glycolysis. Lactate production is also decreased with age in lymphocytes (Lengle *et al.*, 1978). Thus, numerous enzymes are altered with mitogenesis in the aged and these alterations may sufficiently affect requisite biochemical pathways so as to impair the production of essential growth products which are necessary for mitogenesis.

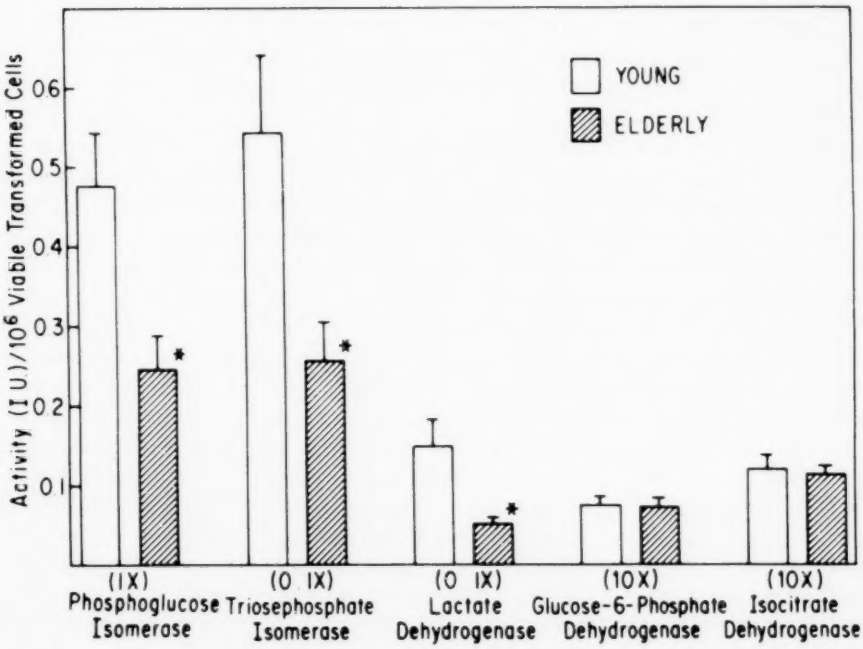

FIG. 5. Histogram of enzyme activity per transformed cell at 72 h of incubation. The actual values for trisephosphate isomrase and lactate dehydrogenase are ten times that depicted and the actual values for glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase are one-tenth that depicted for scaling and comparison purposes. The activity (I.U./ml) divided by the number of viable cells/ml multiplied by the percent transformed cells gives activity (I.U.)/10^6 viable transformed cells. Open bars = young; hatched bars = elderly. Total young subjects = 14; total elderly subjects = 14. Values are means ± SEM. Asterisks represent significant differences ($P \leq 0.05$) between the young and the elderly.

MOLECULAR MECHANISMS

Since requisite biochemical pathways such as glycolysis are impaired during mitogenesis in the aged, it is important to relate these defects not only to the impaired cellular function, but also to more basic defects that may cause the impaired enzyme induction. In fact, Florini (1980) has indicated that particularly fruitful investigations into the aging process would be those that could demonstrate the expression of basic molecular defects through biochemical pathways that cause impaired cellular function and contribute to physiologic decrements of the aged. The model system of mitogenesis is especially useful because this age-altered cellular response relates to the expression of several immune and age-related diseases on the organismic level and is also sensitive to intracellular defects at the biochemical level such as impaired protein synthesis and glycolytic enzyme induction (Tollefsbol and Cohen, 1985a,b). The cause for impaired glycolytic enzyme induction appears to be related at least in part to the decreased protein synthesis during mitogenesis in aged subjects since inhibition of protein synthesis also inhibits glycolytic enzyme induction, DNA synthesis, and mitogenesis mimicing the age-related decrease in these factors (Tollefsbol and Cohen, 1985a). Moreover, changes in isozyme adaptation and the adenine and pyridine nucleotides during mitogenesis in the aged could also contribute to the impaired glycolytic enzyme induction (Van DeGrind, 1982; Chapman *et al.*, 1981, 1983). The cause for the decreased protein synthesis of these cells in the aged could be due to a progressive decrease in the genetic expression of protein synthesis or related to other defects such as an accumulation of altered proteins and decreased protein degradation (Tollefsbol and Cohen, 1985a).

Figure 6 shows a schematic which depicts the summation of intracellular chemical and metabolic defects during mitogenesis in the aged and proposes how basic defects might be expressed through various levels to explain, in part, the age-related impaired mitogenesis and immunosenescence. The more basic defects may not be cell-specific but generally applicable to other cells in the aged as well and underlie cellular senescence. For instance, the model proposes that the decreased protein synthesis of cells in the aged is related to the impaired induction of numerous enzymes requisite for normal cellular function. Since the protein synthetic process itself as well as most other basic responses are dependent upon enzyme function, a "slowing-cycle" effect is proposed whereby a genetically controlled decrease in protein synthesis causes a decrease in enzyme synthesis which further slows enzyme-dependent protein synthesis. The enzyme-dependent processes of DNA synthesis, protein degradation, and cofactor formation are also proposed to contribute to the decrease in protein synthesis with age and the "slowing-cycle" effect (Fig. 6). These basic defects at the genetic, molecular, and biochemical levels are probably not cell-specific but generally applicable to numerous cell types if not all. In the example of the cell-specific immune system of mammals (Fig. 6), these basic defects appear to be expressed through an age-related impaired mitogenesis and contribute to immunosenescence and various immune and age-related diseases. Other physiologic decrements and pathologies of the aged as well as normal aging changes may also be related to the expression of these basic defects through senescence of other cell types.

SUMMARY

There is a decline in immune capacity with age which is expressed on the organismic level by association with numerous immune-related diseases, on the cellular level by im-

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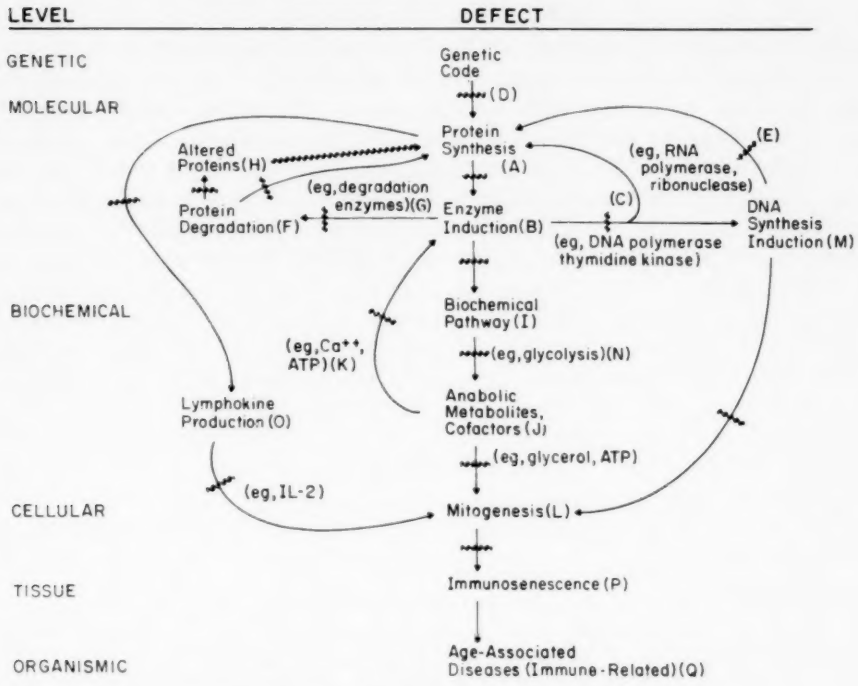

FIG. 6. Model for the expression of basic defects in cellular senescence and aging. Protein synthesis is decreased with age (A) and related to the ability of numerous enzymes to induce (B). A decrease (decreases indicated by wavy lines) in the induction of enzymes necessary for transcription and translation (C) would further decrease protein synthesis creating a "slowing-cycle" effect which is initiated by a progressive decrease in the genetic expression of protein synthesis (D). Other factors may also contribute to the "slowing-cycle" effect such as decreased DNA synthesis (E) and protein degradation (F). The progressive impairment in enzyme induction would be expected to affect protein degradation enzymes (G) causing an accumulation of altered proteins with age (H). The accumulation of altered proteins may contribute to the "slowing-cycle" effect by increasing the molecular entropy of the cell and interfering with inductive mechanisms such as protein synthesis and by decreasing the pool of available amino acids for protein synthesis. The impaired enzyme induction would also be expected to slow the flux of requisite biochemical pathways (I) which are critical to viable cellular functions through the production of anabolic metabolites and cofactors (J). This decrease in cofactors would be expected to further impair the potential for enzyme induction (K) creating an additional "slowing-cycle" effect. These basic defects may represent a general mechanism of aging which is genetically controlled and based on a decreasing protein synthesis causing a "slowing-cycle" effect which eventually renders the cells unable to function. In the example of immunosenescence, these basic defects play a role in cell-specific impaired mitogenesis (L) through a decreased potential for mitosis (M), glycolysis (N), and lymphokine production (O). The impaired mitogenesis in mammals relates to immunosenescence (P) and numerous age-associated diseases (Q) which contribute to the expression of aging.

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paired mitogenesis, on the biochemical level by impaired metabolic pathways, and on the molecular level by decreased protein synthesis and degradation. Defects in various cofactors such as calcium and several nucleotides also occur and may be related to the impaired enzyme function during mitogenesis in the aged. The central cause for decreased mitogenesis in the aged could be a decrease in protein synthesis which appears to cause impaired enzyme induction. This impaired enzyme induction accounts in part for the decreased glycolytic flux and DNA synthesis in these cells. Decreased protein synthesis also has been associated with a decreased synthesis of lymphokines which help these cells to proliferate. Numerous other intracellular age-related defects of lymphocytes also occur which may collectively play important interdependent roles in the impaired lymphocyte function of the aged. A potential general underlying mechanism of cellular senescence is proposed based on a genetic "slowing-cycle" effect of transcription, translation, and enzyme induction with immunosenescence presented as an example of an expression of these basic defects.

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CHANGES IN ACTIVITY AND AMOUNT OF ACTIVE ELONGATION FACTOR 1α IN AGING AND IMMORTAL HUMAN FIBROBLAST CULTURES

JENS CAVALLIUS, SURESH I.S. RATTAN* and BRIAN F.C. CLARK

Department of Chemistry, Aarhus University, DK-8000 Aarhus C, Denmark

Abstract—Stoichiometrically estimated amounts of active elongation factor, EF-1α, remain constant in serially passaged Phase II cultures of human fibroblasts, MRC-5, but decrease by 45% towards the end (Phase III) of their lifespan. Catalytic activity of EF-1α is also reduced by 35% in Phase III old cells. The SV40 transformed immortal cell line MRC-5V2 has 30% higher levels of active EF-1α without significant increase in its catalytic activity. Low-serum-associated G_1 arrest of normal and transformed cells reduces amounts of active EF-1α by 35% and 20%, respectively. Catalytic activity, however, is reduced rapidly only in G_1 arrested normal cells and not in transformed cells. Even though the cell cycle-related changes are reversible both in normal and transformed cells, the age-related decline in amounts of active EF-1α and its activity are irreversible and, most probably, crucial.

Key Words: elongation factor-1α, protein synthesis, aging, immortality, human fibroblasts, stoichiometric assays, catalytic activity

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INTRODUCTION

PROGRESSIVE DECLINE in protein synthesis is one of the few age-related changes observed in almost all aging systems (Makrides, 1983). Mechanistic details of this phenomenon are, however, only beginning to be understood. Studies with nematodes, insects, and rodent tissues and organs indicate that the slowing-down of the elongation phase of protein synthesis is central to this (Bolla and Brot, 1975; Moldave *et al.*, 1979; Gabius *et al.*, 1983; Webster and Webster, 1983, 1984; Blazejowski and Webster, 1983, 1984; Richardson *et al.*, 1984; Egilmez and Rothstein, 1985). Most evidence points specifically towards the elongation factor, EF-1α, as the crucial enzyme in this respect, which loses activity with age (for review, see Webster, 1985). In addition, EF-1α is one of the proteins that are involved in the synthesis of all proteins, including themselves (for review, see Clark, 1980). This not only provides means for testing certain theories of aging, but also is useful for understanding mechanisms of aging at the level of genetic information transfer from nucleic acids to proteins.

*To whom all correspondence should be addressed.
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We have studied changes in amounts and activity of EF-1α during aging of human fibroblasts in culture. The validity of the cell culture as a model for studying aging is well established (Hayflick, 1965; Holliday, 1984), and this provides access to the study of certain aspects of human aging under well-defined conditions. Further, transformed human cells escape aging, possess unlimited proliferative capacity and are generally heteroploid (Hayflick, 1965; Holliday, 1984; Rattan, 1986). They thus offer a comparable system for investigating differences between mortal and immortal cells.

Here we report changes both in the amount of active EF-1α as estimated by stoichiometric assay, and in its catalytic activity during serial passaging of human diploid fibroblast cell strain MRC-5 until the end of its lifespan in vitro, compared with its SV40 transformed immortal cell line, MRC-5V2. The amounts of active EF-1α were estimated by stoichiometric binding of [^{14}C]Phe-tRNA to ribosomes in the presence of GDPNP, a non-hydrolysable analogue of GTP. In the assay for catalytic activity, GTP was used to provide conditions under which EF-1α can function in multiple rounds of aminoacyl-tRNA binding to ribosomes. We have compared both exponentially growing cultures and low-serum-induced quiescent cultures to show age- and cell cycle-related changes in the amounts and activity of EF-1α in normal and transformed cells.

MATERIALS AND METHODS

Cell culture and cell-free extracts

Human embryonic lung fibroblasts, strain MRC-5, and its SV40 virus transformed cell line, MRC-5V2 (Huschtscha and Holliday, 1983), were obtained from Dr. Robin Holliday, NIMR, London. Cells were cultured routinely in Dulbecco's modified Eagle's minimum essential medium (DMEM; Gibco, U.K.) containing 10% fetal calf serum and antibiotics (100μg/ml streptomycin and 100 U/ml penicillin), at 37°C in an atmosphere of 5% CO_2. In vitro lifespan of MRC-5 cells was estimated by calculating the cumulative population doubling level (PDL) attained on 1:2 serial passaging. Before every split, confluent cultures were harvested by trypsinization, resuspended in DMEM, cell number counted in a sample using a Coulter Counter (Coulter Electronics, UK), and the number of PDs the culture had undergone was calculated. In the present series of experiments, starting from PDL 20, MRC-5 cells reached PDL 47, which is here considered as 100% lifespan. As described by Hayflick (1965), cultures were considered to be in Phase II until slowing-down of their growth during the last few passages (Phase III) when they leave the cycle irreversibly. No such change in growth characteristics, however, is observed in serially passaged MRC-5V2 cells, which have unlimited proliferative capacity.

Cell-free extracts for EF-1α assays were prepared as follows. About 4×10^6 cells were washed three times with Hanks' balanced salt solution and collected by scraping off in 0.5 ml of EF-1α protective buffer (25 mM Tris/HCl pH 8, 50 mM KCl, 2 mM benzamidine, 1 mM dithiothreitol, 1 mM PMSF, 0.1 mM EDTA and 25% glycerol). Cells were sonicated at 4°C using Soniprep 150 (MSE, U.K.) at speed 8 for 30 s Nuclei, membranes and mitochondria were removed from the sonicates by centrifugation (45 min, 27,000 g). Protein content in the supernatants was estimated by the Bio-Rad method (Bio-Rad, W. Germany), using BSA as the standard. For assaying EF-1α in different phases of the growth cycle, cultures were considered to be in an exponential phase of growth 20 h after seeding. At this stage, 80–90% of Phase II, less than 20% of Phase III MRC-5 cells and more than

95% of MRC-5V2 cells are capable of entering S phase of the cell cycle as determined by [^{3}H]thymidine labelling and autoradiography (Holliday *et al.*, 1981; Huschtscha and Holliday, 1983). To arrest the cells in G_1 phase of the cell cycle, normal medium was replaced with DMEM containing 0.2% serum for 72 h. Under these conditions, more than 99% of both MRC-5 and MRC-5V2 cells became arrested in G_1 as checked routinely by [^{3}H]thymidine labelling and autoradiography.

Salt-washed ribosomes from Artemia salina *cysts*

Salt-washed ribosomes, free from initiation and elongation factors, were prepared according to Zasloff and Ochoa (1971) and Merrick (1979). Briefly, 200 g cysts were washed with sodium hypochlorite, rinsed with distilled water and homogenised using a N18/N8 Ultra Turax homogeniser (Janke and Kunkel, West Germany) in solution A [35 mM Tris/HCl pH 7.5, 9 mM $Mg(CH_3CO_2)_2$, 70 mM KCl, 0.1 mM EDTA, 10 mM β-mercaptoethanol and 0.25 M sucrose]. The broken cysts were centrifuged for 15 min (15,000 g) to remove debris, nuclei and mitochondria. The supernatant was centrifuged for 25 min (106,000 g) to remove glycogen, which would otherwise sediment with ribosomes. The ribosomal pellet was obtained by centrifugation for 90 min (250,000 g), and was resuspended in solution A without sucrose but with 0.5 M KCl. The suspension was layered over a discontinuous sucrose gradient (0.4 M and 1 M) in solution B [20 mM Tris/HCl pH 7.5, 3 mM $Mg(CH_3CO_2)_2$ and 0.3 M KCl], and centrifuged for 19 hrs (250,000 g). All operations were performed at 4°C. The salt-washed ribosomes were resuspended in solution A to give a concentration of 0.8 $A_{260}/\mu l$, and stored at −80°C.

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Charging of tRNA

Charging of $tRNA^{Phe}$ from yeast was performed essentially as described by Merrick (1979) with the following modifications. The reaction mixture contained in 350 μl: 14.9 nmol [^{14}C]Phe (504 Ci/mol, Amersham, U.K.), 27.2 nmol [^{12}C]Phe, $5 \cdot 10^{-6}$ A_{260} $tRNA^{Phe}$ (Boehringer Mannheim, West Germany), 100 mM Tris/HCl pH 7.5, 50 mM NH_4Cl, 12 mM $MgCl_2$, 2 mM ATP, 2.4 mM CTP, 2.8 mM β-mercaptoethanol and saturating amounts of partially purified yeast aminoacyl-tRNA synthetase (laboratory stock).

The separation of aminoacyl-tRNA from amino acids and synthetases was carried out on Sephadex G-25 (Pharmacia, Sweden) pre-equilibrated and eluted with 5mM sodium acetate (pH 4.8). After ethanol precipitation, the concentration was adjusted to 2 pmol/μl. Counting efficiency was found to be 89.5% in Brays scintillation fluid counted in a liquid scintillation counter (Beckman LS 7500). The specific activity of the Phe was thus found to be 350.5 cpm/pmol Phe.

Ribosome-poly(U)-tRNA complex

The salt-washed ribosomes were pre-incubated with poly(U) and tRNA in the presence of a high concentration of Mg^{2+} in order to avoid the characteristic "lag time" due to the poly(U)-dependent binding of $tRNA^{Phe}$ to the ribosome. The reaction mixture contained in a final volume of 220 μl: 54 mM Tris/HCl pH 7.5, 13 mM $Mg(CH_3CO_2)_2$, 0.1 M NH_4Cl, 37 mM KCl, 0.2 mM DTT, 63 mM sucrose, 2.5 mM β-mercaptoethanol, 0.03 mM EDTA, 875 μg poly(U), 440 μg $tRNA^{Phe}$ and 44 A_{260} salt-washed ribosomes. After incubation at 37°C for 15 min, the mixture was stored on ice until use (normally 1–2 min, maximum 5 min).

Binding assay

The assay was based on Slobin and Möller (1979), Merrick (1979) and Fonzi *et al.*, (1985) with the following modifications. The reaction mixture contained in 50 μl: 60 mM Tris/HCl pH 7.5, 6 mM $Mg(CH_3CO_2)_2$, 20 mM NH_4Cl, 100 mM KCl, 0.7 mM DDT, 13 mM sucrose, 0.5 mM β-mercaptoethanol, 0.6 mM PMSF, 1.1 mM benzamidine, 14% glycerol, 0.06 mM EDTA, 0.01% w/v BSA, 10 pmol [^{14}C]Phe-tRNA^{Phe} (350.5 cpm/pmol), 2 A_{260} ribosome complex and 0.1 mM GDPNP for the stoichiometric assays or 2 mM GTP for the catalytic assays.

In order to avoid hydrolysis to the inhibitor GDP, GDPNP was stored in "use once" aliquots in liquid nitrogen, thawed and added just before incubation in siliconised Eppendorfs at 37°C for 15 min. After incubation, the reaction was stopped by adding 50 μl of 4% glutaraldehyde to stabilise the EF-1α complex bound to the ribosome and collected immediately on nitrocellulose filter (Millipore, type HA pore size 0.45 μm), pre-wetted in wash buffer [20 mM Tris/HCl pH 7.5, 5 mM $Mg(CH_3CO_2)_2$ and 100 mM NH_4Cl]. The filter was washed with 4.5 ml ice cold wash buffer and dissolved directly in 3 ml Brays scintillation fluid and counted in a liquid scintillation counter (Beckman LS 7500). Background was routinely determined and subtracted.

RESULTS

Description of the assays

Under the conditions described in the methods, the amount of [^{14}C]Phe-tRNA bound enzymically to ribosomes was proportional to the amount of EF-1α in the reaction mixture. This correlation was found both for purified EF-1α from mouse liver and for crude cell-extracts prepared after sonication (data not shown). We have also assayed EF-1α using S-100 cell extracts prepared by low speed (25,000 g) and high speed (100,000 g) centrifugation, but found no additional advantage over our method of cell-extract preparation.

Amounts of active EF-1α were estimated by the stoichiometric assay using GDPNP, a non-hydrolysable analogue of GTP, which blocks translocation (Merrick, 1979; Iwasaki and Kaziro, 1979). The catalytic activity of EF-1α was estimated by using GTP at a concentration of 2 mM, which is 3–6 × 10^8 times higher than that of EF-1α in the assay mix (Carvalho *et al.*, 1984). This is because the binding constants of EF-1α for GDP and GTP are very similar as found by us using purified EF-1α from mouse liver (Cavallius *et al.*, in preparation) and from rabbit reticulocytes (Carvalho *et al.*, 1984). Therefore, use of a 10^8-fold higher concentration of GTP eliminates the risk of EF-1α being inhibited by accumulating GDP. This was tested by lowering the concentration of GTP to values 1/100 and 1/1000 as great, which had no effect on the amount of bound Phe in the assay. Both the amount of active EF-1α and its catalytic activity are expressed as pmol [^{14}C]Phe-tRNA bound ribosomes per μg protein. It should be emphasised that the catalytic assay used here does not distinguish between poly(Phe) or single Phe. This means that the observed catalytic activity of EF-1α is a measurement of the combined capacity to bind Phe-tRNA to ribosomes (as in the stoichiometric assay), and synthesis of poly(Phe), which involves EF-1β,α and EF-2 (Iwasaki and Kaziro, 1979). This also gives an overall estimate of the extent of protein synthesis. In addition, although we are not removing cellular ribosomes from the cell extracts, purified ribosomes in complex with poly(U) and tRNA^{Phe} have to be added into the assay-mix to estimate EF-1α activity. The presence of cellular ribosomes in

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the crude extracts does not interfere with the assay, as they are not programmed with poly(U) and therefore do not direct the binding of the radiolabelled aminoacyl-tRNA.

Amounts of active EF-1α

Stoichiometrically estimated amounts of EF-1α in exponentially growing Phase II (up to 85% lifespan completed) MRC-5 cultures remain constant (0.22 pmol/μg protein) during serial passaging, and decrease by 45% in Phase III at the end of lifespan in vitro (Fig. 1A). When MRC-5 cells are arrested in G_1 phase of the cell cycle by low-serum in the culture medium, there is an immediate decrease of 35% in the amounts of active EF-1α. This cell-cycle-related decrease is also observed in old cells in Phase III which already have amounts of EF-1α reduced by 45% owing to aging in vitro (Fig. 1A). Although the cell-cycle-associated changes in the levels of active EF-1α are reversible (up to 50% within 20 h; 100% in 72 h) by restoring serum concentrations in the medium to 10%, age-related decline remains irreversible. In comparison, exponentially growing immortal cells MRC-5V2 have 30% higher levels of active EF-1α (0.29 pmol/μg protein), which are also subject to reduction by 25% due to low serum associated G_1 arrest (Fig. 1B).

Catalytic Activity of EF-1α

Catalytic activity of EF-1α shows similar age- and cell-cycle-related decline in MRC-5 cells, to a slightly lesser extent (Fig. 2A). Exponentially growing Phase II cultures have a constant level (2.24 pmol bound Phe/μg protein) of catalytic activity of EF-1α for 85% of their lifespan in vitro, after which there is a decrease of 35% in Phase III. G_1 arrested Phase II MRC-5 cells have about 25% reduced catalytic activity, whereas Phase III senescent cells, which already have 35% less EF-1α activity owing to aging, show a further

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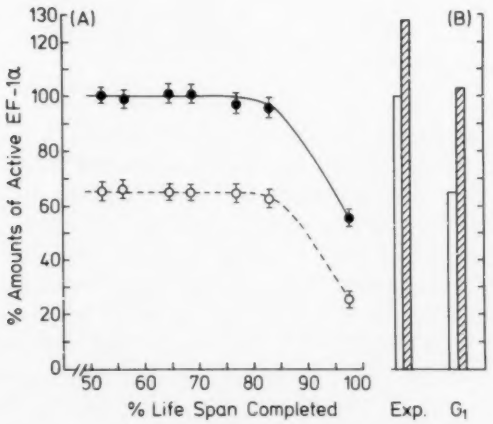

FIG. 1. Stoichiometrically estimated amounts of active EF-1α in cell-free extracts of MRC-5 and SV40 transformed MRC-5V2 cells. (A) Age-related changes in exponentially growing, ●—●; and G_1 arrested MRC-5 cells, ○—○; (B) Comparison between young MRC-5 (open bars), and transformed MRC-5V2 (filled bars) exponentially growing and G_1 arrested cultures. Values are expressed relative to those in young MRC-5 cells, PDL 25 (0.22 pmol/μg protein = 100%), and are ±S.D. of at least four independent measurements.

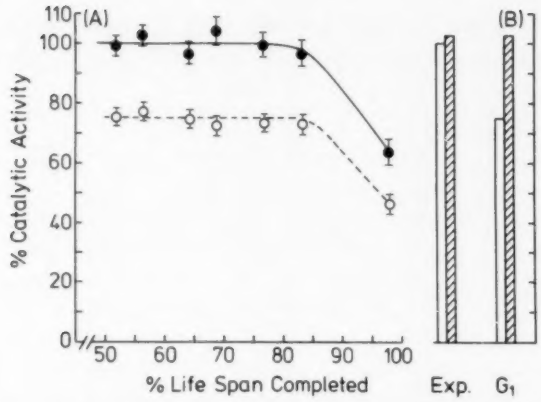

FIG. 2. Catalytic activity of EF-1α in cell-free extracts of MRC-5 and SV40 transformed MRC-5V2 cells. (A) Age-related changes in exponentially growing, ●—●; and G_1 arrested MRC-5 cells ○—○. (B) Comparison between young MRC-5 (open bars), and transformed MRC-5V2 (filled bars) exponentially growing and G_1 arrested cultures. Values are expressed relative to those in exponentially growing young MRC-5, PDL 25 (2.24 pmol bound Phe/μg protein = 100%), and are ±S.D. of at least four independent measurements.

decline of 20% when arrested in G_1 by low serum in the medium, which is a 40% lower level. The cell-cycle-associated changes in the activity of EF-1α are also reversed by 10% serum. In contrast to the differences in amounts of active EF-1α in normal and transformed cells (Fig. 1B), the catalytic activity of EF-1α is almost the same in exponentially growing MRC-5 and MRC-5V2 cells (Fig. 2B). Further, whereas MRC-5 have 25% reduced catalytic activity when kept in low serum for 72 h, MRC-5V2 do not show any such change within this time. After longer periods (about 10 days) of starvation, however, the activity becomes reduced in MRC-5V2 cells also (not shown).

DISCUSSION

Decreased catalytic activity of EF-1α, either in purified form or in cell free extracts, has been reported for old insects, nematodes and rodents (Bolla and Brot, 1975; Moldave *et al.*, 1979; Gabius *et al.*, 1983; Webster and Webster, 1983; Blazejowski and Webster, 1983, 1984). Our studies focus on the measurement of age- and cell-cycle-related changes in catalytic activity and in the amount of active EF-1α in normal human cells which undergo aging in vitro, and in their virus transformed counterparts which are immortal. Using two different binding assays, we have estimated both the amounts of active EF-1α and its activity, so that our study differs from the previous reports on activity alone.

Like many other cellular, biochemical and physiological changes observed in late passage (Phase III) cultures of diploid cells (for review, see Hayflick, 1980), changes in EF-1α also become apparent in Phase III only. Until about 80–85% of their life span in vitro, MRC-5 cells are perfectly able to maintain both the activity and the amounts of active EF-1α, after which there is a sudden decrease. A similar abrupt decline in the level EF-1α, which parallels the decline in total protein synthesis in old insects and rodent organs, has been reported; this provides evidence for the crucial role of EF-1α in aging (Webster, 1985).

The age-related decline in amounts and activity of EF-1α in MRC-5 cells is not attributable simply to the fact that the majority of cells in Phase III are non-dividing (Holliday *et al.*, 1981). This is because even though experimentally induced G_1 arrest reduces both the activity and the amounts of active EF-1α in MRC-5 cells, this change is completely reversible in Phase II cells when normal serum concentration is restored. On the other hand, Phase III cultures, which already have reduced EF-1α, show further reduction due to low serum. This low-serum-associated decline is reversible in old cells also. Of course, the age-related increase in the vulnerability of cells to experimental manipulations (Cristofalo *et al.*, 1985), is also observed in old MRC-5 cells which show relatively more serum-associated decline in EF-1α than Phase II cells. It is, therefore, important to distinguish between "reversible quiescence" due to serum or other factors and "irreversible quiescence" due to intrinsic aging.

Transformed MRC-5V2 cells, however, differ from normal MRC-5 cells both in respect of the amounts of active EF-1α and of their response to low-serum-associated decline. Higher amounts of active EF-1α in exponentially growing transformed cell cultures are not simply due to higher numbers of cycling cells. This is because, when arrested in G_1, MRC-5V2 cells still have higher levels of EF-1α than normal cells in G_1. Even though amounts of active EF-1α are higher in MRC-5V2 cells, catalytic activity shows no increase. Further, low-serum-associated decline in activity is hardly seen in these cells in 72 h even though more than 99% of them become arrested in G_1 during this time, as checked by [^{3}H]thymidine labelling. It is interesting to note that HeLa cells also show no decrease in EF-1 activity in stationary phase (Engelhardt and Sarnoski, 1975). It appears from our data that unlike in normal cells where EF-1α might become a limiting factor for growth and protein synthesis, transformed cells may remain independent of this. It is possible that some other factors with functional homologies with EF-1α, such as the *ras* oncogene product p21 (Clark *et al.*, 1985), compensate for EF-1α. These innate differences in protein synthetic components of normal and transformed cells might be linked intimately to altered control mechanisms and intra- and extra-cellular requirement for unlimited division potential in the latter (Holliday, 1984; Rattan, 1986).

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Our observations on changes in activity and amount of active EF-1α during aging of MRC-5 cells might have various explanations. The presence of some inhibitors causing a reduction in activity is ruled out, as discussed by Engelhardt and Sarnoski (1975), Webster and Webster (1983), and Webster (1985). For example, both the mixing experiments mixing EF-1 active and inactive crude extracts prepared from African Green Monkey kidney cells (Englehardt and Sarnoski, 1975), and addition of cytosol proteins from old *Drosophila* to in vitro assay system (Webster and Webster, 1983) showed no inhibition of peptide chain elongation. The second possibility is that the reduced amounts of active EF-1α and its activity might be due to variations in its synthesis and absolute amounts in terms of the percentage of cellular proteins during aging and different phases of the cell cycle. Age-related decrease in the translatable mRNA for EF-1α in *Drosophila* and mouse organs (Webster and Webster, 1984; Webster, 1985), and its reduced synthesis during different cell cycle phases of CHO cells and African Green Monkey kidney cells (Hassell and Engelhardt, 1976; Fischer *et al.*, 1980), support this view. In human fibroblasts, however, the synthesis of EF-1α as determined by two-dimensional gel electrophoresis does not show cell cycle-, age- or transformation-related variations (Bravo *et al.*, 1983; Rattan *et al.*, 1986). Similarly, no change in EF-1α synthesis is seen during germination of fungal spores, while its activity increases progressively (Fonzi *et al.*, 1985). It is, therefore,

necessary to use better methods such as monoclonal antibodies and immunoblotting to resolve this controversy.

Another explanation which may account for rapid cell cycle changes in amounts of active EF-1α in normal and transformed cells and for its catalytic activity in normal cells during aging can be a post-translational modification such as methylation. In a fungus, *Mucor*, EF-1α methylation and activity are shown to increase concurrently during spore germination (Fonzi *et al.*, 1985). In SV40 transformed mouse fibroblasts higher levels of EF-1α methylation were reported as compared with the permanent cell line 3T3B (Coppard *et al.*, 1983). We have, however, observed no age- or cell cycle-related changes in EF-1α methylation in MRC-5 and MRC-5V2 cells (Rattan *et al.*, 1986).

The fourth possibility which may explain our results is the relationship between multiple forms of EF-1α and its activity. EF-1α exists in heavy and light forms, depending upon whether it is in an aggregated form of chains or not (see Moldave, 1985). The ratio of heavy to light forms is reported to decrease during aging of nematodes and rodent brain (Bolla and Brot, 1975; Vargas and Castañeda, 1981), but not in *Drosophila* (Webster, 1985). During germination of wheat seeds, similar conversion is observed in parallel with a decrease in total protein synthesis (Sacchi *et al.*, 1984). It is, therefore, possible that changes in multiple forms may account for differences in catalytic activity and amounts of active EF-1α in aging and immortal cells. This is particularly so because post-translational conformational changes in a variety of enzymes, including elongation factor 2 in mouse liver, appear to be a general phenomenon during aging (Takahashi *et al.*, 1985; Rothstein, 1985). In addition to the above explanations, our results are also compatible with the view of feedback and accumulation of errors in information transfer pathways resulting in aging and death (see Kirkwood *et al.*, 1984; Rattan, 1985). Since EF-1α is involved in the synthesis of all proteins including itself, a decline in its activity either due to errors or due to one of the many reasons discussed above, might account for the age-related slowing-down of protein synthesis. At this stage, however, we have no direct evidence in support of the error theory.

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TIME COURSE OF THE AGING OF THE HIGH AFFINITY L-GLUTAMATE TRANSPORTER IN RAT CORTICAL SYNAPTOSOMES

D.D. WHEELER* and J.G. ONDO

Department of Physiology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, South Carolina 29425

Abstract—Initial velocities of cortical synaptosomal glutamate uptake are measured as a function of both glutamate and sodium concentration in 10- and 18-month old animals and compared to previous results from 2- and 30-month old animals. As a percentage of 2-month values, mean velocity of uptake falls to 87% in the 10-month group and to 79% in the 18-month group, with no further change between 18 and 30 months. The data give minimal best fit to the same model for interaction of carrier with glutamate and sodium as found previously for 2- and 30-month old animals. Thus the basic mechanisms involved in transport do not change with age. However, the kinetic parameters which describe the transporter do change with age, the primary changes being in those parameters reflective of the transport capacity of the carrier. *Va*, apparent maximal velocity of uptake, declines most rapidly between 2 and 30 months, continues to decline rapidly between 10 and 18 months, and then declines slowly between 18 and 30 months. *Jm*, rate of uptake at infinite sodium concentration, declines most rapidly between 2 and 10 months, with a much slower decline between 10 and 30 months. *Kt*, apparent Michaelis-Menten constant, changes but little with age; at 30 months, *Kt* is only 6–11% less than at 2 months. Although the total change is only 9–10%, there appears to be a slow, steady decline in K_{Na}, the sodium concentration giving an uptake equal to *Jm*/2, between 2 and 30 months of age.

Key Words: aging, time course, glutamate transport, kinetics, rat, synaptosomes

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INTRODUCTION

GLUTAMIC ACID is one of several amino acids thought to function as synaptic transmitters in the central nervous system (Wheeler, Boyarsky, and Brooks, 1966, Krnjevic, 1974; Roberts, Storm-Mathisen, and Johnston, 1981). Essential to glutamate's transmitter function is the operation of a high affinity transport mechanism (Wheeler and Boyarsky, 1967; 1968; Logan and Snyder, 1971; 1972), which serves to concentrate the glutamate in the pre-synaptic terminals and to remove and thus inactivate the glutamate following stimulus-induced release. Uptake by this mechanism is almost totally dependent on the presence of sodium in the incubation medium (Navon and Lajtha, 1969; Bennett, Logan, and Snyder, 1973; Wheeler and Hollingsworth, 1978).

*Address reprint requests to Dr. Wheeler at the above address.
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Kinetic studies of the sodium dependence of synaptosomal glutamate uptake which have been carried out in this laboratory indicate that uptake takes place via the following mechanism (Wheeler, 1979):

$$\begin{array}{ccccccc} & & & \overset{K_4}{} & & \overset{K_5}{} & \\ C & + \mathrm{Na} & \rightleftharpoons & C\mathrm{Na} & + \mathrm{Na} & \rightleftharpoons & C\mathrm{Na}_2 \\ + & & & + & & & + \\ G & & & \mathrm{G} & & & \mathrm{G} \\ \Updownarrow K_1 & & \overset{K_2}{} & \Updownarrow & & \overset{K_3}{} & \Updownarrow \\ C\mathrm{G} & + \mathrm{Na} & \rightleftharpoons & C\mathrm{NaG} & + \mathrm{Na} & \rightleftharpoons & C\mathrm{Na}_2\mathrm{G} \\ & & & \downarrow k_6 & & & \downarrow k_7 \end{array}$$

$$V = \frac{\dfrac{K_6[\mathrm{Na}][\mathrm{G}]}{K_1K_2} + \dfrac{K_7[\mathrm{Na}]^2[\mathrm{G}]}{K_1K_2K_3}}{1 + \dfrac{[\mathrm{G}]}{K_1} + \dfrac{[\mathrm{Na}][\mathrm{G}]}{K_1K_2} + \dfrac{[\mathrm{Na}]^2[\mathrm{G}]}{K_1K_2K_3} + \dfrac{[\mathrm{Na}]}{K_4} + \dfrac{[\mathrm{Na}]^2}{K_4K_5}}$$

in which C = carrier; Na = sodium; G = glutamate; K_1–K_5 = dissociation constants; k_6, k_7 = translocation constants; $K_6 = k_6[C]t$; and $K_7 = k_7[C]t$. Although sodium is necessary for uptake, concentrations of sodium above certain levels (depending on the glutamate concentration) are inhibitory to uptake compared to the optimum sodium concentration.

Uptake has also been found to be dependent on the membrane potential (Wheeler and Graves, 1983). Modelling studies of the membrane potential dependence suggest that the energy for glutamate transport comes from the electrochemical potential gradient for sodium and glutamate. Deductions from these studies of membrane potential combined with those from the kinetic studies are consistent with the operation of a symmetrical carrier; that is, the above reaction scheme may be repeated in reverse order on the inside of the membrane. Furthermore, the results are consistent with the operation of a neutral carrier, which when loaded with two sodium and one glutamate ions has a net positive charge. Thus inward translocation of the CNa_2G form of the carrier is facilitated by the membrane potential. Such a symmetrical carrier might also function in glutamate release, since depolarization of the membrane would shift the equilibrium toward the outside of the membrane.

In previous aging studies, significant changes were found to have occurred in the synaptosomal glutamic acid transporter when 30-month old animals were compared to 2-month old animals (Wheeler, 1980). Initial velocity of glutamate uptake was measured as a function of both glutamate and sodium concentration. At all combinations of glutamate and sodium concentrations used, uptake was less in the 30-month age group.

The data were fitted to models which describe the mechanism by which carrier, glutamate, and sodium interact in the process of transport. Apparently, there is no change with age in the basic mechanism of transport, since data from 30-month old animals gave minimal best fit to the same model as found for 2-month old animals (see model above). However, changes were found to have occurred in the best fit constants which quantitate

the model. Consequently, there are a number of changes in the parameters which characterize the transporter. In general, those parameters indicative of maximal transport capability declined with age: (1) *Va*, apparent maximal velocity of uptake, declined by 28% at physiological sodium concentration; and (2) *Jm*, hypothetical uptake at infinite sodium and a given glutamate concentration, declined by 28–33%. However, those parameters indicative of carrier affinities changed less with age: (1) *Kt*, the apparent Michaelis-Menten constant, declined by about 10% at physiological sodium concentration; and (2) K_{Na}, sodium concentration giving a velocity of uptake equal to *Jm*/2, declined by about 10%.

In the present report, we have extended these studies to 10-month and 18-month old animals and compared the results to those described above for 2- and 30-month old animals in an attempt to define the time course of the aging of this transport mechanism.

METHODS

The methods used in this study have been published previously (Wheeler, 1980). Only a summary is given below.

Male Long-Evans rats 10 or 18 months of age were housed two per cage under conditions of constant temperature and controlled illumination, with *ad lib.* access to food and water. The diet was obtained from Wayne Feeds (Allied Mills, Inc., Libertyville, IL).

Preparation of synaptosomes

Animals were sacrificed by decapitation and the brains were rapidly removed and placed on glass dissection plates on ice. The cortices were then rapidly dissected free and homogenized in 20 volumes of ice cold 0.32 M sucrose. The homogenates were centrifuged for 10 min. at 1085 × g (2°C) to remove nuclei and cellular debris. The synaptosomes were then sedimented by centrifugation at 27,000 × g for 15 min. The supernatant was discarded, and the synaptosomes were resuspended in cold sucrose.

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Measurement of uptake

Uptake of 14-C labelled L-glutamic acid was measured by incubation of synaptosomes for one minute in a modified Krebs-Henseleit solution at 30°C (Lockwood, 1961). All incubations were carried out in a Dubnoff metabolic shaker. The concentration of glutamate in the final incubation medium was 5.0, 2.0, 1.0, 0.6667, or 0.5 × 10^{-5}M. Concentration of sodium in the incubation medium was varied by replacement of NaCl with equiosmolar Tris buffer; 19.0, 27.0, 43.0, and 120.8 mM sodium solutions were used with each of the above glutamate concentrations. Following incubation, synaptosomes were recovered on Millipore filters, washed, and the filters blotted and counted for radioactivity. Single measurements of initial velocities for each of the glutamate concentrations, along with each of the several sodium concentrations, were made with synaptosomes from a single preparation of tissue from one animal. The measurements were repeated on a total of six animals for each age group.

Previous electronmicrographic studies have shown that the process of separating synaptosomes from incubation medium by use of Millipore filters concentrates the synaptosomal fraction (Collings, Braid, Greene, and Wheeler, 1986). Furthermore, autoradiographic studies showed that the synaptosomes account for 81% or more of the glutamate taken up (Collings *et al.*, 1986).

Computer optimization

After correction for filter retention of label when incubation medium is poured through them, initial velocities were calculated, and these data were fitted to the velocity equation. A parameter optimization algorithm based on the Flexible Polyhedron approach of Nelder and Mead (1965) was used to minimize the following function:

$$J = \sum_{i=1}^{n} \left(\frac{V_{\text{model}} - V_{\text{data}}}{V_{\text{data}}} \right)^2$$

J, the objective function, is the sum of the squared fractional errors between the velocities of uptake predicted by the equation (V_{model}) and those observed experimentally (V_{data}).

Statistical Analysis

Analysis of variance was carried out with the use of Human Systems Dynamics' ANOVA II program and the Apple II+ computer.

RESULTS

Comparison of initial velocities

Initial velocity of uptake was measured as a function of both sodium and glutamate concentration in synaptosomes from 10-month and 18-month old animals. As a percentage of 2-month values, mean velocity of uptake fell to 87% by the age of 10 months and to 79% by 18 months (Table 1). However, there was no further decline from 18 to 30 months. The decline in initial velocity with age is highly significant; multi-way analysis of variance gave a P value less than 0.001 (Table 2). The analysis also showed significant ($P < 0.001$) sodium/age interaction and glutamate/age interaction. This implies that the carrier interactions with both sodium and substrate change with age. The interaction between sodium and glutamate is also highly significant ($P < 0.001$); however, this interaction does not change significantly with age ($P = 0.069$). An ANOVA was also done between the 2-month age group and each of the other ages. For all ages, the differences are highly significant; $P = 0.002$, 0.001, and 0.001 for the 10-, 18-, and 30-month age groups, respectively.

Best fit model

The data for 10-month old and 18-month old animals were fitted to the model found to give minimal best fit to data from both 2-month old animals and 30-month old animals (See Introduction). The rate equation for this model can be rearranged as follows:

$$v = \frac{\dfrac{K_3K_6[\text{Na}] + K_7[\text{Na}]^2}{K_2K_3 + K_3[\text{Na}] + [\text{Na}]^2}[\text{G}]}{\dfrac{K_1K_2K_3K_4K_5 + K_1K_2K_3K_5[\text{Na}] + K_1K_2K_3[\text{Na}]^2}{K_2K_3K_4K_5 + K_3K_4K_5[\text{Na}] + K_4K_5[\text{Na}]^2} + [\text{G}]} = \frac{Va[\text{G}]}{Kt + [\text{G}]} \quad (1)$$

For both age groups, the model gives a good fit to the data; for the 10-month group, the average percent error between experimental data and model predictions is only 2.20%,

TABLE 1. COMPARISON OF INITIAL VELOCITIES

[Na], mM	[G] × 10^5M	2 Months	10 Months	18 Months	30 Months
19	0.5	0.47 ± .01	0.42 ± .02 (0.89)	0.36 ± .02 (0.77)	0.38 ± .02 (0.81)
	0.6	0.60 ± .01	0.53 ± .02 (0.88)	0.45 ± .02 (0.75)	0.47 ± .03 (0.75)
	1.0	0.79 ± .02	0.69 ± .03 (0.87)	0.64 ± .04 (0.81)	0.69 ± .10 (0.87)
	2.0	1.23 ± .03	1.09 ± .05 (0.89)	0.96 ± .05 (0.78)	0.98 ± .05 (0.80)
	5.0	1.93 ± .06	1.73 ± .06 (0.90)	1.54 ± .09 (0.73)	1.47 ± .09 (0.76)
Mean Ratio			(0.886)	(0.768)	(0.798)
27	0.5	0.69 ± .02	0.60 ± .03 (0.87)	0.57 ± .03 (0.83)	0.55 ± .03 (0.80)
	0.6	0.81 ± .03	0.72 ± .03 (0.89)	0.68 ± .04 (0.84)	0.66 ± .03 (0.81)
	1.0	1.09 ± .03	0.95 ± .04 (0.87)	0.91 ± .05 (0.83)	0.93 ± .05 (0.85)
	2.0	1.61 ± .05	1.37 ± .06 (0.85)	1.33 ± .08 (0.83)	1.30 ± .06 (0.81)
	5.0	2.27 ± .06	2.06 ± .08 (0.91)	1.85 ± .11 (0.81)	1.85 ± .10 (0.81)
Mean Ratio			(0.878)	(0.828)	(0.816)
43	0.5	0.95 ± .02	0.85 ± .03 (0.89)	0.81 ± .05 (0.85)	0.79 ± .04 (0.83)
	0.6	1.14 ± .04	1.01 ± .04 (0.89)	0.95 ± .05 (0.83)	0.91 ± .04 (0.80)
	1.0	1.48 ± .03	1.24 ± .05 (0.84)	1.22 ± .07 (0.82)	1.14 ± .06 (0.77)
	2.0	2.04 ± .07	1.76 ± .07 (0.86)	1.61 ± .10 (0.79)	1.61 ± .09 (0.79)
	5.0	2.80 ± .09	2.36 ± .09 (0.84)	2.14 ± .12 (0.76)	2.07 ± .13 (0.74)
Mean Ratio			(0.864)	(0.810)	(0.786)
120.8	0.5	1.24 ± .03	1.10 ± .04 (0.89)	0.95 ± .04 (0.77)	0.99 ± .05 (0.80)
	0.6	1.53 ± .04	1.22 ± .03 (0.80)	1.14 ± .06 (0.75)	1.12 ± .06 (0.73)
	1.0	1.81 ± .05	1.56 ± .04 (0.86)	1.37 ± .08 (0.76)	1.39 ± .08 (0.77)
	2.0	2.24 ± .08	1.82 ± .08 (0.81)	1.69 ± .11 (0.75)	1.70 ± .07 (0.76)
	5.0	2.76 ± .10	2.23 ± .10 (0.81)	2.07 ± .13 (0.75)	2.01 ± .11 (0.73)
Mean Ratio			(0.834)	(0.756)	(0.758)
Overall Mean Ratio			(0.866)	(0.791)	(0.790)

Values shown are mean ± S.E.M. Values in parentheses are expressed as a fraction of the 2-month values. N = 12, 6, 6, and 7, for the 2-, 10-, 18-, and 30-month age groups, respectively. Data for 2- and 30-month age groups have been published previously (Wheeler, 1980).

TABLE 2. ANOVA OF 4 × 4 × 5 FACTORIAL DESIGN FOR AGE, SODIUM CONCENTRATION, AND GLUTAMATE CONCENTRATION

Source	SS	DF	MS	F	P
Between Groups					
Age	9.920	3	3.307	9.449	<0.001
Error	9.441	27	0.350		
Within Groups					
Na	45.265	3	15.088	718.476	<0.001
Age × Na	1.063	9	0.118	5.619	<0.001
Error	1.670	81	0.021		
Glu	134.712	4	33.678	1603.714	<0.001
Age × Glu	1.954	12	0.163	7.762	<0.001
Error	2.292	108	0.021		
Na × Glu	1.813	12	0.151	30.200	<0.001
Age × Na × Glu	0.250	36	0.007	1.400	0.069
Error	1.542	324	0.005		

Human Systems Dynamics ANOVA II program was used in conjunction with an Apple II+ computer to do the analysis of variance. Glu = Glutamate.

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compared to 2.41% for the 18-month age group. Any attempt to simplify the model reduced the goodness of fit. Thus these results confirm the previous conclusion (Wheeler, 1980) that the basic mechanism by which glutamate and sodium interact with carrier and are taken up does not change with age. However, as indicated by the differences in initial velocities, and as will be shown below for the kinetic parameters which quantitate the model, there are quantitative changes with age. These changes are reflected in the best fit constants for the model (Table 3).

The rate equation for the minimal best fit model can be used along with the constants to define and compute certain parameters which may be used in comparing the transporter in the different age groups (Wheeler, 1979; 1980).

Va, apparent maximal velocity of uptake

At infinite glutamate concentration, the rate equation reduces to the following:

$$v = K_3K_6[\mathrm{Na}] + K_7[\mathrm{Na}]^2/(K_2K_3 + K_3[\mathrm{Na}] + [\mathrm{Na}]^2) = Va \tag{2}$$

Thus *Va* is a measure of the transport capacity at a given sodium concentration. At all sodium concentrations used, *Va* declines with age (Fig. 1). The rate of decline is greatest between 2 and 10 months of age, slows down between 10 and 18 months, and is slowest between 18 and 30 months. The initial rate of decline increases with the [Na], being slowest with 19 mM [Na] and fastest with 140 mM [Na]. At 30 months of age, the ratio (as a percentage) of *Va* to that at 2 months is 78, 76, 74, 72, and 72 for [Na]'s of 19, 27, 43, 120.8, and 140 mM, respectively.

Jm, rate of uptake at a given glutamate concentration and infinite sodium concentration

When only the sodium concentration is infinite, the rate equation reduces to the following:

$$v = \frac{K_7[\mathrm{G}]}{\dfrac{K_1K_2K_3}{K_4K_5} + [\mathrm{G}]} = Jm \tag{3}$$

Thus *Jm* is a measure of the capacity of the carrier at high sodium concentration. *Jm*

TABLE 3. CONSTANTS GIVING BEST FIT

K	2 M	10 M	18 M	30 M
1	3.7132×10^{-5}	4.6814×10^{-5}	5.3561×10^{-5}	3.8726×10^{-5}
2	1.0448×10^{1}	1.0543×10^{1}	1.0132×10^{1}	1.5530×10^{1}
3	4.3981×10^{-5}	3.4847×10^{-5}	2.5894×10^{-5}	2.6171×10^{-5}
4	3.3579×10^{-1}	2.8022×10^{1}	2.3049×10^{1}	5.4387
5	8.3615×10^{-3}	1.136×10^{-4}	9.9536×10^{-5}	5.0730×10^{-4}
6	1.5827×10^{3}	1.7037×10^{3}	1.4107×10^{3}	1.962×10^{3}
7	2.6678	2.0476	2.0974	1.9152

Where *K*'s represent V_{max}'s (K_6, K_7) units are *n*-mol/10 mg • min.

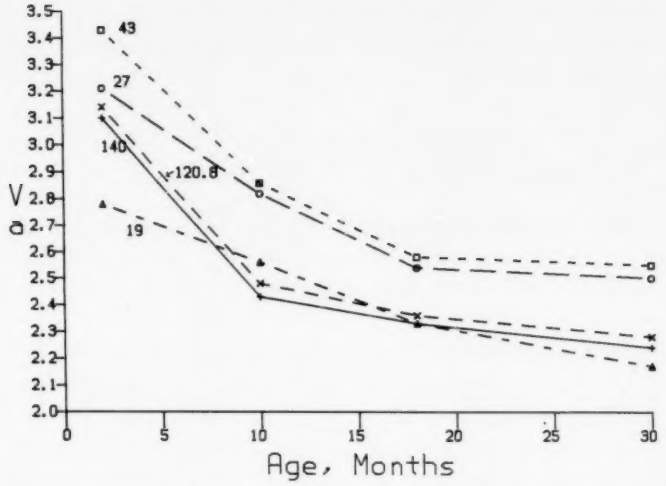

FIG. 1. *Va*, apparent maximal velocity of uptake, as a function of age. *Va* was calculated by use of Eq. 2 and the constants of Table 3. Sodium concentration (mM) is given by each curve. Units of *Va* are n-mol/10 mg tissue minute.

declines with age for all glutamate concentrations used (Fig. 2). As was seen for *Va*, the steepest decline is between 2 and 10 months; after 10 months, the decline is much slower and relatively linear. *Jm* at 30 months is 74, 74, 73, 73, and 72% of that at 2 months for glutamate concentrations of 0.5, $0.\bar{6}$, 1.0, 2.0, and 5.0×10^{-5} M, respectively.

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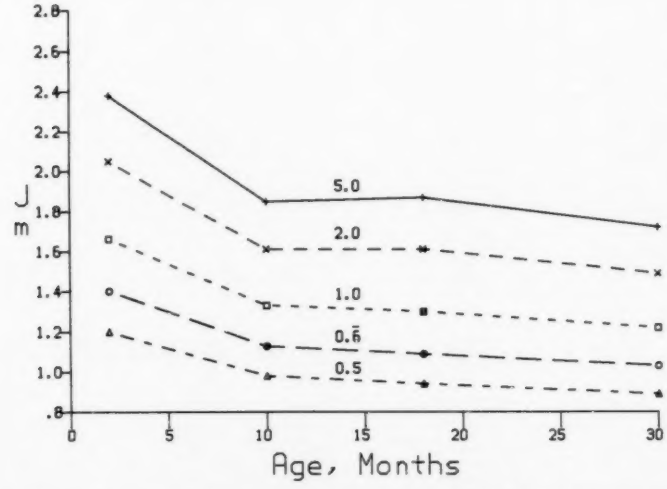

FIG. 2. *Jm*, uptake at a given glutamate concentration and infinite sodium concentration, as a function of age. Jm was calculated by use of Eq. 3 and the constants of Table 3. Glutamate concentration $\times 10^5$ M, is given above each plot. Units of *Jm* are n-mol/10 mg tissue minute.

Kt, the apparent Michaelis-Menten constant

Kt, the glutamate concentration which gives a velocity of uptake equal to *Va*/2, is defined by the model as follows:

$$Kt = \frac{K_1K_2K_3K_4K_5 + K_1K_2K_3K_5[\mathrm{Na}] + K_1K_2K_3[\mathrm{Na}]^2}{K_2K_3K_4K_5 + K_3K_4K_5[\mathrm{Na}] + K_4K_5[\mathrm{Na}]^2} \quad (4)$$

Although relatively small, there appears to be a slight decline in Kt with age (Fig. 3); Kt at 30 months is 94, 92, 89, 89, and 90% of that at 2 months. This implies that there may be a small increase with age in the apparent affinity of the carrier for glutamate.

K_{Na}, the sodium concentration which gives a velocity of uptake equal to Jm/2

Whereas *Kt* is a measure of the apparent carrier affinity for glutamate, K_{Na} is a measure of the apparent affinity for sodium. When the velocity of uptake is equal to *Jm*/2, then K_{Na} is equal to the sodium concentration as defined by the rate equation. Under these conditions, the rate equation can be rearranged into the form of a quadratic equation for [Na] as follows:

$$[\mathrm{Na}]^2(K_1K_2K_3K_7 + K_4K_5K_7[\mathrm{G}]) + [\mathrm{Na}](K_1K_2K_3(2K_3K_6 - K_5K_7) + K_3K_4K_5[\mathrm{G}](2K_6 - K_7)) - K_2K_3K_4K_5K_7([\mathrm{G}] + K_1) = 0 \quad (5)$$

For each glutamate concentration, the coefficients for the quadratic equation were calculated and K_{Na} determined from the real roots of the solution of the equation (Fig. 4). For all glutamate concentrations, the differences in K_{Na} between age groups are small. However, there appears to be a slight decline with age; in all cases, K_{Na} for a given age group is less than the corresponding K_{Na} for the 2-month age group. K_{Na}'s for the

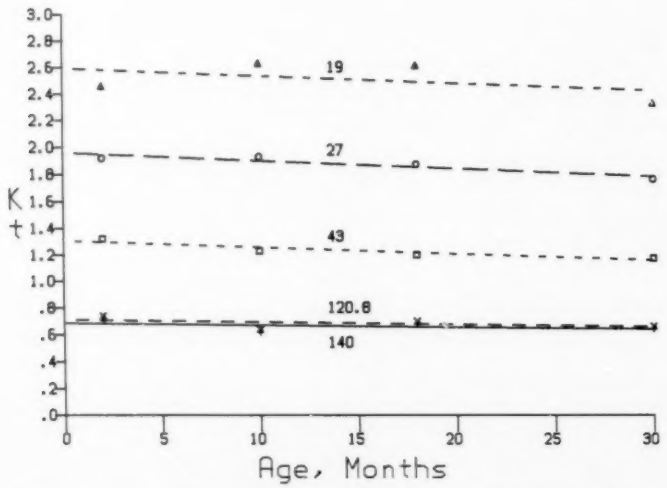

FIG. 3. *Kt*, apparent Michaelis-Menten constant, as a function of age. *Kt* was calculated by use of Eq. 4. Sodium concentration (mM) is given above each plot. Units of *Kt* are 10^{-5} M.

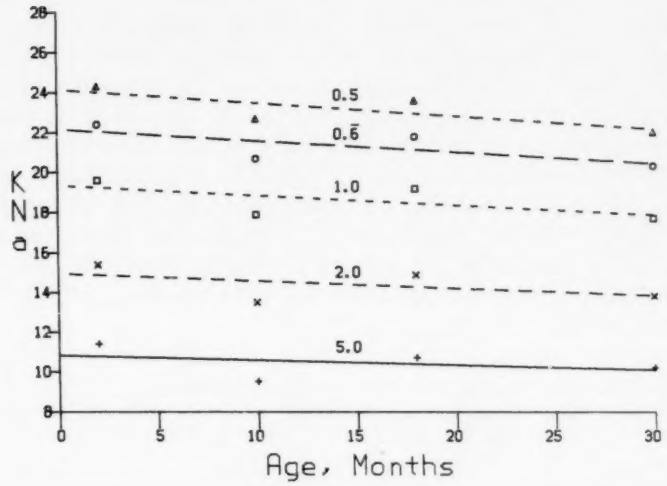

FIG. 4. K_{Na}, the sodium concentration giving an uptake equal to $Jm/2$, as a function of age. K_{Na} was calculated from Eq. 5. Glutamate concentration $\times 10^4$ M, is given above each plot. Units of K_{Na} are mM.

30-month age group are 9–10% less than that for the 2-month age group. Thus there may be a small increase with age in apparent affinity for sodium.

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DISCUSSION

For all age groups, the same minimal best fit model resulted from the modelling studies. This supports previous conclusions (Wheeler, 1980) that the fundamental mechanics of the carrier do not change with age.

However, changes were found to occur with age in the constants which quantitate the carrier. As a result, measured velocity of uptake declined with age for all combinations of glutamate and sodium concentration used. The mean velocity of uptake declines by about 13% between 2 and 10 months of age and by another 8% between 10 and 18 months. There was no further decline in mean velocity between 18 and 30 months of age, although the kinetic parameters continued to change somewhat.

The changes with age in the best fit constants are also reflected in the kinetic parameters. Those parameters indicative of the carrier capacity, *Va* and *Jm*, were found to change most with age. Although both of these parameters continue to decline between 18 and 30 months of age, most of the decline occurs prior to the age of 18 months. For *Jm*, most of the decline occurs between 2 and 10 months of age, while *Va* continues to decline fairly rapidly from 10 to 18 months of age, although not as rapidly as between 2 and 10 months of age.

Those changes reflective of apparent carrier affinity change much less with age, although both *Kt* and K_{Na} are 6–11% less in the 30-month age group than in the 2-month age group. Thus there may be a small increase in carrier affinity for both glutamate and sodium in the 30-month age group, although marginal at best.

The glutamic acid transporter is essential to the function of glutamic acid as a transmitter. Both concentration of the transmitter in the presynaptic terminals and re-uptake

following stimulus induced release are functions of the transporter. Thus a reduction with age in the capability of this transporter might be expected to have deleterious effects on synaptic transmission. Both a reduction in the amount of glutamate released (as a consequence of a lower concentration in the terminal) and slowing of the rate of its inactivation are possible consequences. Decline in transmitter concentration has been confirmed in a previous study (Wheeler and Ondo, 1986). Endogenous synaptosomal GABA concentration was found to have declined when 30-month old animals were compared to 2-month old animals. Since the nervous system plays such a major role in homeostasis of the entire organism, alteration with age in synaptic function might be expected to have far-ranging consequences (Franks, 1970; Sohal and Sharma, 1972; Hayflick, 1976; Diamond, 1978; Lytle and Altar, 1979).

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BRAIN DOPAMINE AUTORECEPTORS IN AGING RATS

N. CARFAGNA, F. TRUNZO and A. MORETTI*

Farmitalia Carlo Erba Research Center, 20014 Nerviano, Italy

Abstract—The function of dopamine (DA) autoreceptors is evaluated in vivo in striatum and mesolimbic regions of young adult (4 months), mature (14 months) and old (26 months) male Wistar rats. γ-Butyrolactone (GBL)-induced dihydroxyphenylalanine (DOPA) accumulation in rats also treated with an inhibitor of aromatic L-aminoacid decarboxylase was used to determine the presence of synthesis-modulating nerve terminal autoreceptors while its reversal with apomorphine served as an index of autoreceptor stimulation. GBL-induced DOPA accumulation in striatum is very high at all three ages (130–150% increase in comparison with controls) as is its reversal by apomorphine (65–80% decrease in comparison with GBL alone). In mesolimbic regions, GBL has much less effect than in striatum (31% rise at 4 and 26 months, 12% rise at 14 months), but apomorphine's effect is of the same order of magnitude (down 60–80%). The conclusion can be drawn that aging does not significantly affect DA autoreceptor function in striatum and mesolimbic areas.

Key Words: aging, dopamine autoreceptors, rat brain

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INTRODUCTION

AGING IS associated with an impairment of monoaminergic, especially dopaminergic neurotransmission in humans and various animal species (for reviews see Samorajski, 1977; Pradhan, 1980; McGeer, 1981; Joseph and Roth, 1983; Rogers *et al.*, 1984). Reduction of the content (Carlsson and Winblad, 1976; Finch, 1978; Strong *et al.*, 1982; Algeri *et al.*, 1983; Simpkins, 1984), biosynthetic enzyme activities (McGeer and McGeer, 1978), synthesis (Finch, 1978, Algeri *et al.*, 1983), turnover (Carfagna *et al.*, 1985), receptor density measured in vitro (Memo *et al.*, 1980; Misra *et al.*, 1980; De Blasi and Mennini, 1982; Roth, 1982; Severson *et al.*, 1982) and in vivo (Wong *et al.*, 1984) and adenylate cyclase activity (Memo *et al.*, 1980; Govoni *et al.*, 1977; Makman et al., 1980) were described in various dopamine-rich brain areas. Thus, aging affects the dopamine (DA) system both pre- and post-synaptically. This can lead to deterioration of various neurophysiological functions such as motor activity, hormone secretion, temperature control, sleep etc. Moreover, the resulting imbalance with other neurotransmitter systems, for example serotonin and acetylcholine, could explain the pathogenesis of various pathological states in old age (Burchinsky, 1985).

*Address correspondence and reprint requests.
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In this context, very little information is available on age-related changes of DA autoreceptors. This term "autoreceptors", coined by Carlsson (1975), refers to receptors located on the soma and/or dendrites and on the axon terminals. DA autoreceptors are involved in the physiological activity and pharmacological responsiveness of DA-containing neurons. Nerve terminal DA autoreceptors modulate both the synthesis and release of DA while those located on the soma/dendrites influence the dopaminergic firing rate coupled to synthesis and release (reviewed by Roth, 1984). DA autoreceptors are more sensitive to DA agonists than postsynaptic receptors. DA agonists not only inhibit the firing rate of DA neurons in the substantia nigra (Bunney and Aghajanian, 1975; Aghajanian and Bunney, 1977; Skirboll *et al.*, 1979) but also DA synthesis and release from nerve terminals (Walters and Roth, 1976; Kehr *et al.*, 1977; Di Chiara *et al.*, 1978; Starke *et al.*, 1978; Marek and Roth, 1980; Hjorth *et al.*, 1981; Argiolas *et al.*, 1982; Haubrich and Pflueger, 1982; Demarest *et al.*, 1983; Grabowska Andén and Andén, 1984; Fadda *et al.*, 1984) and these effects are antagonized by neuroleptics. Consequently, autoreceptor stimulation reduces, while inhibition increases the overall influence of DA systems on postsynaptic cells.

Available findings (Scalzo and Spear, 1985) suggest that DA autoreceptor function is reduced in mesolimbic areas of mature (12–13 months) compared to young (2–3 months) rats. No information is available about older animals.

In the present paper DA autoreceptors were studied in the striatum and mesolimbic areas of young adult (4 months), mature (14 months) and old (26 months) rats. A well established biochemical technique was applied which involves measuring in vivo catecholamine synthesis as 3,4-dihydroxyphenylalanine (DOPA) accumulation after treatment with γ-butyrolactone (GBL, which blocks impulse flow in DA neurons, and stimulates DA synthesis: Gessa *et al.*, 1968; Spano *et al.*, 1971; Walters and Roth, 1976) in rats also treated with 3-hydroxybenzylhydrazine (NSD 1015, to inhibit L-aromatic aminoacid decarboxylase, Carlsson *et al.*, 1972) and analyzing the reversal of this accumulation by pretreatment with apomorphine. GBL induces changes in the properties of tyrosine hydroxylase that result in removal of the feedback regulation. DA synthesis is thus enhanced as revealed by DOPA accumulation; this increase is reversed by treatment with agonists such as apomorphine.

MATERIALS AND METHODS

Animals

Male Ico:WI (IOPS AF/Han) rats aged 4, 14 and 26 months were obtained from Iffa-Credo, Les Oncins, France. Their median life is 24 months. None of them had visible tumors. They were housed at 22 ± 1°C with a 12-hour light/dark cycle (6 a.m.–6 p.m.) and were allowed free access to water and standard diet (Sourriffarat, Iffa-Credo) with 23% protein content.

Drug treatment

NSD 1015, 100 mg/kg i.p. was administered 30 min before decapitation. As described in the classical GBL model, GBL, 750 mg/kg i.p. and apomorphine (APO), 2 mg/kg s.c. in 0.1% ascorbate, were injected respectively 5 and 15 min before NSD 1015.

Tissue sampling

Rats were decapitated by guillotine. Striatum and mesolimbic areas (n. accumbens and tuberculum olfactorium) were rapidly dissected according to Glowinski and Iversen (1966) and Waldmeier and Maître (1976), respectively, frozen on dry ice and stored at −80°C until analysis.

DOPA determination

Tissue samples were homogenized in cold 0.1 N perchloric acid containing 3,4-dihydroxybenzylamine (DHBA) as internal standard. DOPA was extracted by absorption on activated alumina (Anton and Sayre, 1962), the eluate was filtered (MF-1 BAS) and DOPA was assayed by HPLC with electrochemical detection (Nissinen and Taskinen, 1982). A Waters model 590 pump with a 5 μm ODS BAS column fitted with a C_{18}-Corasil Waters precolumn was used. Electrochemical detection was done using a glassy carbon electrode (LC-4B, BAS) operating at + 0.7 V. The recovery of DHBA throughout the whole procedure was 72.0 ± 1.9% (striatum) or 80.3 ± 2.3% (mesolimbic areas). Each data was obtained from one animal's tissues.

Statistical analysis

Two-way (treatment and age) analysis of variance (ANOVA) (Huitson, 1966) was done followed by Kramer's test (Kramer, 1956) for comparison of treatment groups within each age (see Figures).

This analysis was done in the Farmitalia Carlo Erba Biometric Department.

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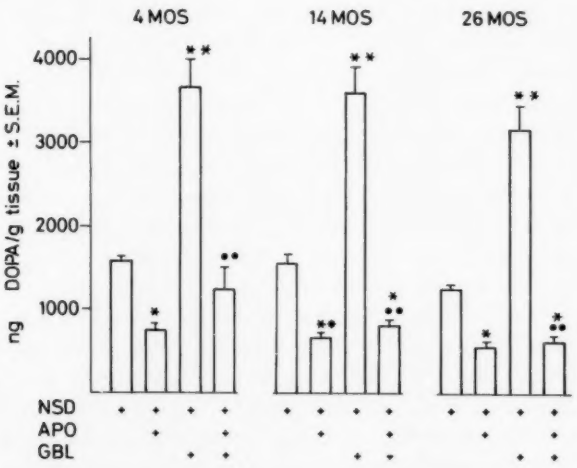

FIG. 1. DOPA accumulation in the striatum of 4, 14 and 26-month-old Wistar male rats after various treatments. NSD 1015 (100 mg/kg i.p.) was administered 30 min before decapitation. GBL (750 mg/kg i.p.) and APO (2 mg/kg s.c.) were injected respectively 5 and 15 min before NSD 1015. Values are presented as means for group of 8–16 rats and vertical bars denote 1 SEM. * $p < 0.05$ or ** $p < 0.01$ compared to control group (NSD alone); •• $p < 0.01$ compared to NSD/GBL group. ANOVA results: Age $F(2,127) = 3.0$; $p < 0.10$. Treatment $F(3,127) = 112.3$; $p < 0.01$. Age × Treatment $F(6,127) = 0.2$; $p > 0.05$.

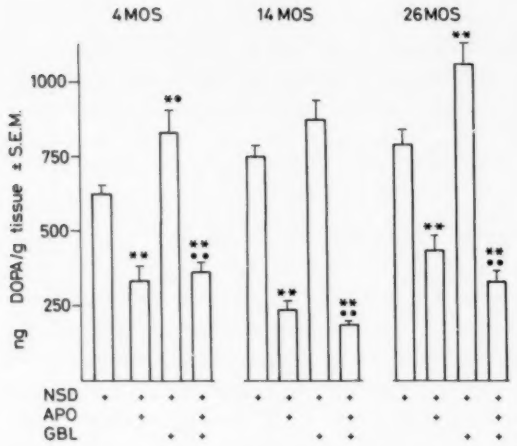

FIG. 2. DOPA accumulation in the mesolimbic areas of 4, 14 and 26-month-old Wistar male rats after various treatments. NSD 1015 (100 mg/kg i.p.) was administered 30 min before decapitation. GBL (750 mg/kg i.p.) and APO (2 mg/kg s.c.) were injected respectively 5 and 15 min before NSD 1015. Values are presented as means for groups of 8–16 rats and vertical bars denote 1 SEM. ** $p < 0.01$ compared to control group (NSD alone); •• $p < 0.01$ compared to NSD/GBL group. ANOVA results: Age $F(2,120) = 7.7$; $p < 0.01$. Treatment $F(3,120) = 85.7$; $p < 0.01$. Age × Treatment $F(6,120) = 1.6$; $p > 0.05$.

RESULTS

ANOVA results indicate no treatment × age effect in either area of rat brain (see bottom of Figs. 1 and 2). Comparison among treatments was made by Kramer's test. Fig. 1 shows that the basal accumulation of DOPA (NSD alone) in striatum was the same in young adult (4 months) and mature (14 months), but slightly less in the old ones (26 months) (this difference was not significant). APO reduced DOPA accumulation by 55% (mean of three ages): 52% in young adult, 57% in mature, 56% in old rats. In the NSD-treated rats, GBL markedly increased DOPA accumulation by a mean of 135%: (+130% in comparison with NSD alone in young adult, +129% in mature, +156% in old rats). APO inhibited GBL's effect as shown by 76% decrease in the NSD/APO/GBL group in comparison with NSD/GBL (66% in young adult, 78% in mature, and 80% in old rats). All these effects of GBL and APO were statistically significant or highly significant.

Fig. 2 shows the results in the mesolimbic areas. The basal DOPA values were much lower than in striatum with a slight, nonsignificant tendency to increase at 14 and 26 months compared with 4 months. The APO-induced reduction was of the same order of magnitude as in striatum (mean 54%) and greater in mature (68%) than in young adult (50%) or old rats (45%). However, the GBL stimulating effect was much lower than in striatum: +31% in young adult and old rats (both significant), but only 12% (nonsignificant) in mature rats. Again, APO strongly inhibited GBL's effect to a highly significant extent (mean −68% in NSD/APO/GBL group in comparison with NSD/GBL), −60% in young adults, −75% in mature, and −68% in old rats.

DISCUSSION

In the present paper DA autoreceptor function was studied with a biochemical approach involving the inhibition by GBL of impulse flow in DA neurons and the reversal

by APO of the resulting DOPA increase as an index of autoreceptor stimulation. The effects of APO alone, GBL alone and APO + GBL were measured on DOPA accumulation in striatum and mesolimbic areas of rats treated with NSD 1015 as well to inhibit DOPA decarboxylase.

Regional distribution of autoreceptors in young rats

The 52% inhibition of DA synthesis by APO in both areas reported in the present paper agrees with previous data (Walters and Roth, 1976; Demarest *et al.*, 1983). GBL-induced DOPA accumulation in striatum (+130%) is similar to that found by some Authors (Demarest *et al.*, 1983; Grabowska Andén and Andén, 1984) but lower than reported by others (Walters and Roth, 1976; Marek and Roth, 1980; Haubrich and Pflueger, 1982; Fadda *et al.*, 1984).

GBL-induced DOPA accumulation in mesolimbic areas is known to be less pronounced than in striatum and the literature has dealt with the possible reasons for the differences (Demarest *et al.*, 1983; Roth, 1979; Andén *et al.*, 1983). Some Authors even found no effect of GBL on DA synthesis (Lawson *et al.*, 1981) or DOPAC levels (Beart and Gundlach, 1980) in the nucleus accumbens.

Our findings (32% increase after GBL) confirm the reduced sensitivity of mesolimbic areas in comparison with striatum. They agree with one report (Kehr *et al.*, 1977) but are lower than others (Marek and Roth, 1980; Hjorth *et al.* 1981; Demarest *et al.*, 1983; Scalzo and Spear, 1985). Either strain or dissection differences could account for this quantitative disagreement. The observation that in the two cases in which GBL had less effect on mesolimbic areas rats were of Wistar strain, while larger increase was found in Sprague-Dawley rats, may be more than a coincidence.

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Interestingly, the presence of DA autoreceptors in a third DA system, the mesocortical, is still controversial (Roth, 1984; Fadda *et al.*, 1984).

Despite the above differences in the sensitivity of striatum and mesolimbic areas to GBL, the administration of APO with GBL caused a similar 60–65%-reduction of DOPA accumulation in both areas in comparison with GBL alone. This finding is comparable with published data (Marek and Roth, 1980; Demarest *et al.*, 1983; Scalzo and Spear, 1985).

Taken together, these results suggest that in both areas DA autoreceptors are similarly sensitive to activation by APO.

Effect of aging on DA autoreceptors

GBL-induced DOPA accumulation in striatum (130–150%) and its reversal by APO (66–80%) were similar at 4, 14 and 26 months. In mesolimbic areas GBL had the same effect (31%) except in 14-month animals (12%). In spite of this, the APO's decreasing effect was of the same order of magnitude in all three age groups (60–75%).

The only published data on the age-related changes of DA autoreceptors (Scalzo and Spear, 1985) are difficult to compare with ours because they concerned young (2–3 months) and mature (12–13 months) animals only, and DA levels were assayed (DOPA decarboxylase was not blocked). Different sensitivity has been shown when DA instead of DOPA is taken as a measure in the GBL model (Feenstra *et al.*, 1983). However, it is interesting that in the above paper the reactivity of mesolimbic areas to GBL was reduced at 12–13 months in comparison with 2–3 months, a finding which has now been confirmed by us.

Apart from the latter result which concerns maturity, our work clearly indicates that senescence has no effect on the function of DA autoreceptors as shown by their sensitivity to treatment with GBL and/or APO either in striatum or mesolimbic areas.

It is pertinent to recall that DA synthesis estimated in striatum and nucleus accumbens by measuring DOPA accumulation after inhibition of DOPA decarboxylase, is unchanged in old and young rats (Demarest *et al.*, 1980). Furthermore, neither DA accumulation and release in vitro (Thompson *et al.*, 1981), nor the storage mechanism of brain monoamines (Ponzio *et al.*, 1984) is modified in the aging brain.

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INTRA-REGIONAL VARIATIONS IN THE EFFECT OF AGING ON HIGH AFFINITY CHOLINE UPTAKE, CHOLINE ACETYLTRANSFERASE AND MUSCARINIC CHOLINERGIC RECEPTORS IN RAT NEOSTRIATUM

RANDY STRONG,†‡ CAROL REHWALDT,† and W. GIBSON WOOD†‡

Abstract—High affinity sodium-dependent choline uptake (HACU), choline acetyltransferase (ChAT) and quinuclidinyl benzilate binding ([^{3}H]-QNB) are measured in synaptosomal preparations from four areas of the neostriatum of Fischer 344 rats of three different ages (6, 18 and 30 months). There is a marked regional distribution of all three markers, being higher generally in lateral as compared to medial striatal regions. In addition, in the medial neostriatum, all three measures are higher rostrally than caudally. HACU is reduced with age in the rostromedial and the caudolateral neostriatum. Small (usually less than 20%) but significant decreases in muscarinic cholinergic receptors occur in all regions of the neostriatum. There are no significant age-associated differences in ChAT activity in any region. The lack of decrease in ChAT is evidence that the reductions in HACU in striatal subregions are not simply the result of a loss of axon terminal integrity. The changes in HACU may reflect altered activity of cholinergic neurons in specific striatal subregions.

Key Words: neostriatum, high affinity choline uptake, choline acetyltransferase, muscarinic cholinergic receptors, quinuclidinyl benzilate binding

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INTRODUCTION

IMPAIRED NEUROTRANSMISSION in the brain, as evidenced by neurochemical alterations, may underly some of the behavioral dysfunctions that occur during aging (Ingram *et al.*, 1981; Joseph *et al.*, 1978; Marshall and Berrios, 1979; Strong *et al.*, 1980). It is now well established that the severity of age-related alterations in neurochemical parameters is regionally variable and that within a brain region some parameters may be more affected than others (see Pradhan, 1980). This selectivity manifested by the aging process can be of help in elucidating the neurochemical bases of the decline of behaviors associated with a given brain region. In this regard, the caudato-putamen (neostriatum), as compared to other brain regions, seems especially vulnerable to alterations in pre- and postsynaptic

†Geriatric Research, Education and Clinical Center, Veterans Administration Medical Center, St. Louis, Missouri 63125.
‡Department of Internal Medicine, St. Louis University School of Medicine, St. Louis, Missouri 63125
Address correspondence and reprint requests to: R. Strong, Ph.D., GRECC (111G-JB), VA Medical Center, St. Louis, MO 63125.
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cholinergic neurochemical parameters (Strong *et al.*, 1980; Allen *et al.*, 1983; Morin and Wasterlain, 1980; Waller *et al.*, 1983). These changes may be related to age-associated declines in behaviors that require an intact neostriatal cholinergic system (Strong *et al.*, 1980; Finch *et al.*, 1981). Furthermore, the effects of aging on the cholinergic system vary between subregions of this brain area. We have previously reported that age-related decreases in choline acetyltransferase (ChAT) activity and muscarinic cholinergic receptor ([^{3}H]-quinuclidinyl benzilate, [^{3}H]-QNB) binding were localized to certain discrete subregions while other neostriatal regions were relatively unaffected (Strong *et al.*, 1982, 1984a).

These findings are noteworthy in light of the known neurochemical, pharmacological and functional heterogeneity of the cholinergic system within the neostriatum (Graybiel *et al.*, 1981; Fonnum *et al.*, 1978; Guyenet *et al.*, 1977; Scally *et al.*, 1978; Prado-Alcala *et al.*, 1981; Rea and Simon, 1981). Interruption of cholinergic neurotransmission by microinjections of kainic acid or anticholinergic drugs has been reported to produce different behavioral effects depending on the site of injection within the neostriatum. Effects localized to specific striatal sites include deficits in active or passive avoidance (tests of learning and memory), sensorimotor disturbances and impaired limb use (Dunnet and Iversen, 1982; Neill and Grossman, 1970; Prado-Alcala *et al.*, 1981). These behavioral disturbances also occur in aged rodents (Marshall, 1982; Bartus *et al.*, 1982; Wallace *et al.*, 1980).

The present series of experiments was designed to localize age dependent changes in cholinergic parameters within the neostriatum of Fischer 344 rats. Although we had previously compared ChAT activity and [^{3}H]-QNB binding in aging Sprague Dawley rats (Strong *et al.*, 1982, 1984a) we reexamined these parameters in the Fischer 344 rat strain because it has been reported that age differences in these parameters may be strain and species dependent (Waller *et al.*, 1983; Strong *et al.*, 1980, Reiss *et al.*, 1977). In addition to these cholinergic markers, sodium-dependent, high affinity choline uptake (HACU) was also measured. This parameter was studied because it may be rate limiting in the formation of acetylcholine (ACh) (Simon *et al.*, 1976). Furthermore, HACU in vitro may be used as a measure of the activity of cholinergic neurons in vivo (Atweh *et al.*, 1976). ChAT activity, [^{3}H]-QNB binding and HACU were measured in synaptosomal preparations from medial and lateral portions of successive coronal slices of the neostriatum. The results of these experiments reveal that there are age-associated alterations in pre- and postsynaptic cholinergic neurochemical parameters in the neostriatum of Fischer 344 rats. Furthermore, the data provide evidence that alterations in HACU in discrete striatal subregions are not the result of degenerative changes in cholinergic axon terminals.

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MATERIALS AND METHODS

Animals

Male Fischer 344 rats 6 months (young), 18 months (middle aged), and 30 months (old) of age were used in the following experiments. These rats were obtained from National Institute on Aging contract colonies at Charles River Breeding Laboratories (Wilmington, MA) and Harlan Sprague Dawley Incorporated (Indianapolis, IN) and kept for two months prior to sacrifice. The animals were housed singly under barrier conditions in a Lab

Products Animal Stay Clean Unit (Lab Products, Inc., Maywood, NJ) which provided a unidirectional laminar flow of air that was filtered to remove particles 0.3 μm and larger. The animals were maintained on a 12 h light/dark cycle (7 a.m. to 7 p.m. light) with free access to food and water.

Tissue preparation

Three rats, one rat from each group, were used each day. To control for possible differences owing to circadian rhythms, the order in which the age groups were sacrificed was changed daily. Each rat was killed by decapitation, the brain quickly removed, placed in ice-cold sucrose and coronal sections (2 mm thick) containing the neostriatum were cut anterior and posterior to the anterior commissure. The neostriatum on each side of the brain was divided into equal medial and lateral parts and carefully dissected from the surrounding tissue. The corresponding medial or lateral regions in a coronal section were pooled bilaterally and homogenized in ice-cold 0.32 M sucrose using 10 strokes of a Teflon homogenizer (Wheaton Instruments) on setting 3.8. A synaptosomal pellet (fraction P_2), obtained by differential centrifugation (Gray and Whittaker, 1962), was gently resuspended in 20 volumes of ice-cold 0.32 M sucrose. Protein was measured (Lowry *et al.*, 1951) using bovine serum albumin as standard.

High affinity [^{3}H]-choline uptake

Sodium-dependent high affinity choline uptake was determined using a modification (Pedata *et al.*, 1980) of the procedure of Simon *et al.*, (1976). A modified Krebs-Henseleit buffer was used containing the following (in mM): glucose (10), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (20) NaCl (150), KCl (6.2), MgSO4 (1.2) and $CaCl_2$ (2). A sodium free buffer was made by substituting 300 mM sucrose for NaCl. While on ice, the P_2 suspension of each striatal region (50 μl) was added to 12 × 75 mm test tubes in triplicate containing 450 μl of ice-cold oxygenated buffer and 0.5 μM [^{3}H]-choline chloride. The reaction was initiated by vortexing and placing each tube in a 30°C water bath. Each tube was incubated for 5 min. Initial experiments had established that uptake was linear for at least 7 min using the methods reported here. The reaction was terminated by rapid filtration through Millipore filters (0.65 μm) immediately followed by rinsing each filter with 10 ml of Na^+-free buffer. To correct for nonspecific attachment and passive diffusion, a duplicate set of tubes (blanks), containing tissue and the Na^+-free buffer, was treated as above. For each animal, the time from sacrifice to the completion of the assay was approximately 90 min. Each filter was placed in a liquid scintillation vial and 1 ml of distilled water was added to lyse the synaptosomes. After 1 h, 10 ml of liquid scintillation fluid (Aquasol, New England Nuclear Corp.) was added to each vial, the samples were placed on a shaker for an additional hour then refrigerated overnight and radioactivity was estimated by liquid scintillation spectroscopy. The remaining P_2 homogenate from each sample was stored at −70°C until assayed for ChAT and [^{3}H]-QNB.

Cholinergic muscarinic receptor binding

Each sample was brought up to 60 volumes (original wet weight of tissue) with ice-cold 50mM Na^+-K^+ phosphate buffer (pH 7.4), sonicated for 3 s and centrifuged at 48,000 × g for 10 min at 4°C. The pellet was resuspended in 40 volumes ice-cold buffer by sonication

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and recentrifuged. The final pellet was resuspended in 40 volumes of ice-cold buffer and receptors were assayed according to the method of Yamamura and Snyder (1974). Each sample was assayed in triplicate by adding 50 μl of tissue suspension to test tubes on ice containing 850 μl ice-cold Na^+-K^+ buffer (pH 7.4) and 100 μl of [^{3}H]-QNB (1 nM final concentration). Non-specific binding was determined in an identical set of tubes containing 0.1 μM unlabelled atropine. The tubes were vortexed and placed back in ice and the reaction was initiated by placing the tubes in a 25°C water bath. After 60 min the reaction was terminated by filtering all tubes simultaneously over glass filters (Whatman GF-B) using a cell harvester (Brandell Inc., Gaithersburg, MD). Each filter was washed with 15 ml of ice-cold buffer. Radioactivity remaining on the filters was estimated by liquid scintillation spectroscopy.

Choline acetyltransferase assay

Triton X-100 was added to the remaining homogenate, to give a final concentration of 0.1% and resonicated briefly. A portion of this homogenate was assayed for protein by the method of Lowry *et al.*, (1951). ChAT was assayed in 20 μl of homogenate in duplicate as previously described by Fonnum (1975).

Statistical analysis

All statistical analyses were performed using the Systat statistical package (Systat Inc., Evanston, IL) on an IBM PC computer system. For each neurochemical parameter, means calculated for the different age groups in each region were compared using a two-way analysis of variance of three ages by four regions. The significance of differences between individual means was assessed by Duncan's New Multiple Range Test (Duncan, 1957). Differences were deemed significant when $p < 0.05$. Correlation coefficients (r) were also calculated to determine linear relationships among neurochemical parameters and ages.

RESULTS

High affinity choline uptake

Figure 1 demonstrates the uneven regional distribution of HACU in striatal regions. A two-way analysis of variance of age and region indicated that there was a significant effect of region ($p < 0.001$, df 3,78; $F = 105.338$) and age ($p < 0.025$, df 2,78; $F = 3.887$) but no significant interaction between age and region on HACU. In all groups, HACU was significantly higher in lateral as compared to medial striatal regions. This disparity between medial and lateral striatum was greatest in the caudal region where HACU was approximately 4 times greater laterally than medially. In addition, there were significant rostral-caudal differences in the medial striatum with HACU in the rostral region being about twice that in the caudal region. There were no rostral-caudal differences in the lateral striatum except for the oldest age-group. Figure 1 and Table 1 demonstrate the association between age and HACU. There was a significant ($p < 0.025$) negative correlation between HACU and age in the rostromedial and caudolateral striatum (Table 1). Marked decreases (27%) occurred as early as 18 months of age and no further decrease at 30 months (Fig. 1). In addition, a significant decrease occurred in the 30-month-old group

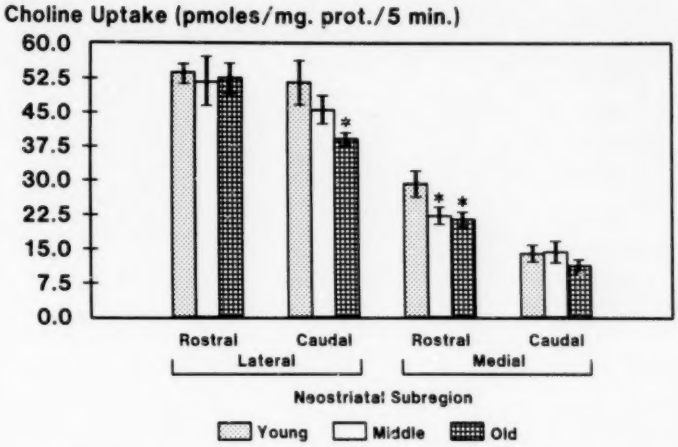

FIG. 1. Distribution of sodium-dependent, high affinity choline uptake in rat neostriatum. Young, middle, and old groups were 6, 18, and 30 months of age, respectively. Values are the mean ± S.E.M. of 7–8 individual determinations performed in triplicate. All medial-lateral differences were significant ($p < 0.05$). All rostro-caudal differences in medial striatum were significant ($p < 0.05$). (*Significantly different from the 6 month old group, $p < 0.05$).

as compared to the 6-month group, but not as compared to the 18-month group in the caudolateral striatum (Fig. 1).

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Cholinergic Receptor Binding

The regional distribution of specific binding (Fig. 2) was also uneven. A two-way analysis of variance of age by region indicated that there was a significant effect of region ($p < 0.001$, df 3,72; $F = 91.25$) and age ($p < 0.001$, df 2,78; $F = 18.808$) but no significant interaction between age and region. In all age groups, there were significant rostral-caudal differences in the medial striatum, which is similar to the distribution of HACU. Additionally, there were no rostral-caudal differences in the lateral striatum. In contrast to HACU, there were no medial-lateral differences in the rostral striatum. There were, however, significant medial-lateral differences in the caudal region with binding being

TABLE 1. CORRELATIONS BETWEEN AGE AND CHOLINERGIC PARAMETERS IN NEOSTRIATAL REGIONS

	Neostriatal Region			
Parameter	Rostromedial	Rostrolateral	Caudomedial	Caudolateral
ChaT	−0.161 (24)	0.032 (24)	−0.366 (24)	−0.337 (24)
HACU	−0.498* (22)	−0.051 (23)	−0.202 (22)	−0.466* (23)
^{3}H-QNB	−0.552**(21)	−0.571**(21)	−0.556**(21)	−0.644**(21)

Each value is a correlation coefficient derived from the number of comparisons shown in parentheses.
*$p < 0.025$.
**$p < 0.01$ (two tailed test).

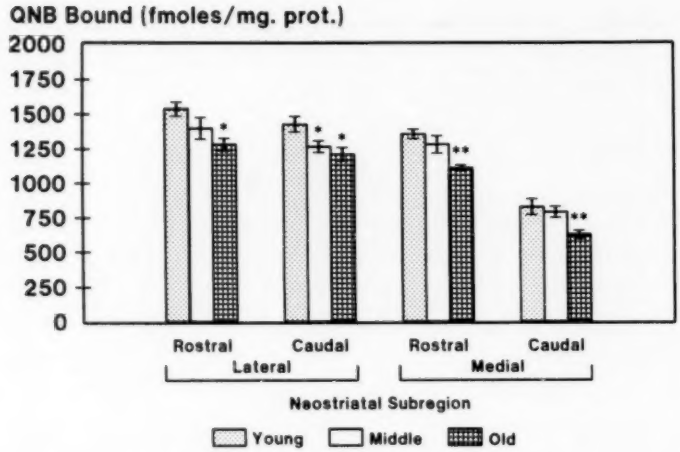

FIG. 2. Distribution of cholinergic muscarinic receptor binding in the rat neostriatum. Young, middle, and old groups were 6, 18, and 30 months of age, respectively. Values are the mean ± S.E.M. of 7 individual determinations performed in triplicate. All lateral medial differences were significant ($p < 0.05$) in the caudal striatum. All rostral-caudal differences were significant ($p < 0.05$) in medial striatum. (* Significantly different from the 6-month-old group, $p < 0.05$. **Significantly different from the 6 and 18-month-old groups, $p < 0.05$.)

about 40% higher in lateral striatum. Table 1 shows the association between age and [^{3}H]-QNB binding in each striatal subregion. Significant ($p < 0.01$) negative correlations of binding with age occurred in all regions of the striatum. In the two lateral regions and in the rostromedial region, the difference between the oldest and youngest group was 15–20% (Fig. 2). In the caudomedial region, the difference between the means of the oldest and youngest group was a little larger (25%) (Fig. 2). A significant ($p < 0.05$) difference between the 18-month-old group and the youngest group was detected in only one region, the caudolateral striatum (Fig. 2).

Choline Acetyltransferase

There was a significant effect of region (df 3,84; $F = 110.614$, $p < 0.001$) on ChAT activity. In all age groups it was significantly higher laterally than medially and there was a significant rostral-caudal difference in the medial striatum with ChAT being two-fold higher in the rostral striatum. Figure 3 and Table 1 show that there was no association between age and ChAT activity. There were no significant differences between means of age groups in any neostriatal region (Fig. 3).

A comparison of all neurochemical parameters within each age group is shown in Table 2. All correlations were positive and highly significant ($p < 0.01$).

DISCUSSION

The present study is in agreement with several previous studies which demonstrated that the cholinergic system in the rat neostriatum is unevenly distributed throughout the nucleus (Graybiel *et al.*, 1981; Takano *et al.*, 1980; Scally *et al.*, 1978; Takano *et al.*, 1981). In general, the density of cholinergic innervation was greatest in the lateral half of

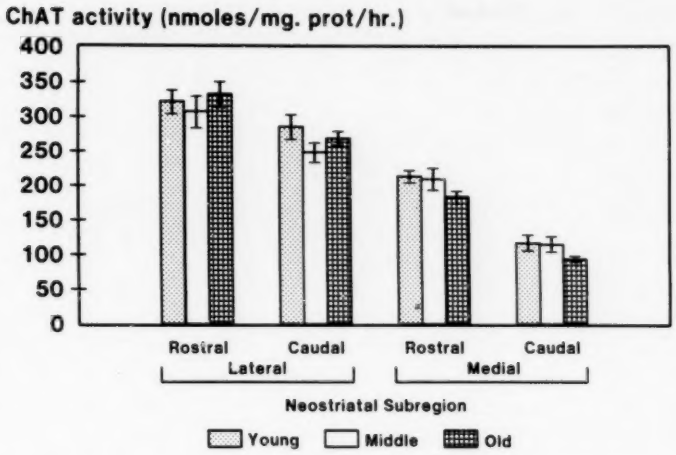

FIG. 3. Distribution of choline acetyltransferase activity in rat neostriatum. Young, middle, and old groups were 6, 18, and 30 months of age, respectively. Values are the mean ± S.E.M. of 8 individual determinations performed in duplicate. All medial-lateral differences were significant ($p < 0.05$). All rostral-caudal differences were significant in the medial striatum ($p < 0.05$).

the neostriatum. In addition, in the medial portion of the striatum, all of the cholinergic markers were higher rostrally than caudally. This rostral-caudal gradient was not evident for any of the cholinergic markers in the lateral striatum. These results are in agreement with previous reports on the distribution of ChAT and [^{3}H]-QNB (Rea and Simon, 1981; Strong *et al.*, 1982, 1984a). Furthermore, the patterns of distribution of ChAT and HACU were 2 to 3 times higher laterally than medially in both rostral and caudal neostriatum whereas the lateral-medial gradient of [^{3}H]-QNB binding was only observed in caudal striatum. These results of the simultaneous determination of ChAT, HACU and [^{3}H]-QNB are in substantial agreement with those of Rea and Simon (1981).

Each parameter of the cholinergic system exhibited a different pattern with respect to the effects of aging in neostriatal subregions. The effect of aging on HACU was regionally dependent with significant decreases (25–30%) occurring in the rostromedial and

TABLE 2. CORRELATIONS BETWEEN CHOLINERGIC PARAMETERS WITHIN AGE-GROUPS

	Ages					
	Young (6 mo.)		Middle (18 mo.)		Old (30 mo.)	
Parameter:	QNB	HACU	QNB	HACU	QNB	HACU
ChAT	0.874	0.679	0.878	0.815	0.870	0.842
HACU	0.716	–	0.693	–	0.689	–

Each value is a correlation coefficient calculated from 24 comparisons except in the 18 month group where each value was derived from 23 comparisons. All correlations are significant at $p < 0.01$

caudolateral regions. In contrast to this marked regional dependency of age-associated changes in HACU, small (usually less than 20%) but significant decreases in [^{3}H]-QNB binding occurred in all regions of the neostriatum. On the other hand, no significant age-related differences in ChAT activity were detected in any region.

The finding of significant age-related decreases in [^{3}H]-QNB binding is in accord with studies of whole neostriatum from aged Wistar rats (Morin and Wasterlain, 1980), Sprague Dawley rats (Strong *et al.*, 1980) and C57Bl mice (Strong *et al.*, 1980; Waller and London, 1983). The finding of no regional effects of aging on muscarinic receptor binding in the present work differs somewhat, however, from our previous study of receptor binding in neostriatal subregions of Sprague Dawley rats (Strong *et al.*, 1984a). In that study there were marked regional variations in the occurrence of significant age-differences in density of muscarinic receptors. Furthermore, age-related decreases in some regions approached 50%, in contrast to the less than 20% reductions found in the present study. As the ages of animals employed in the two studies were almost identical, this may reflect differences in the effect of aging on the two rat strains.

The lack of age-associated changes in ChAT in neostriatal subregions in Fischer 344 rats in the present study is in accord with a previous study measuring no change in ChAT in whole striatum from this rat strain (Reis *et al.*, 1977). However, this finding is in contrast to several previous studies employing other rat strains. Aging was shown to result in decreases in ChAT in grossly dissected neostriatum (Meek *et al.*, 1977; Strong *et al.*, 1980) and in tissue from discrete neostriatal subregions of Sprague Dawley rats (Strong *et al.*, 1982). Most studies of whole striatum of Wistar rats have also found age-differences in ChAT activity (McGeer *et al.*, 1971; Lai *et al.*, 1981; Allen *et al.*, 1983) although one study did not (Morin and Wasterlain, 1980). While some of these discrepancies may be the result of differences in methodology, previous studies comparing different strains of mice or different species of rodents strongly suggest that the effect of aging on ChAT may be related to genotype (Waller *et al.*, 1983; Strong *et al.*, 1980; Reis *et al.*, 1977).

The lack of change in ChAT in the present study is taken as evidence that the regionally selective decreases in HACU are not simply secondary to the age-associated reductions in neuron cell bodies and axon terminal synapses that have been reported to occur in the striatum of Fischer 344 rats (Brizzee *et al.*, 1981). The age-related alteration in HACU in the present study may, on the other hand, reflect a regionally selective decrease in the activity of cholinergic interneurons in vivo. It is well established that HACU *in vitro* is affected by experimental procedures that alter the activity of cholinergic neurons in vivo. Thus, it was suggested that *in vitro* HACU may be used as a measure of cholinergic activity in vivo (Atweh *et al.*, 1976; Kuhar and Murrin, 1978). The changes in HACU in the present study may reflect decreases in the activity of cholinergic neurons in response to regionally selective changes in afferent neurotransmitter systems that project to those neurons. In this regard, we have previously measured alterations in dopamine and norepinephrine tissue content and high affinity uptake of GABA and glutamate in discrete striatal subregions of aged Sprague Dawley rats (Strong *et al.*, 1982, 1984b). The possibility that age-associated changes in one or more striatal afferent neurotransmitter systems may be related to decreases in HACU, as observed in the present work, deserves further study.

There is increasing evidence that disruption of cholinergic function in discrete regions of the rat neostriatum has specific behavioral consequences. Microinjection of atropine

into the anterior caudate resulted in deficits of retention of avoidance learning whereas no behavioral changes were observed in rats injected in other neostriatal regions or in the parietal cortex (Prado-Alcala *et al.*, 1981). Similarly, microinjections of the neurotoxin kainic acid into the mid-ventral region of the neostriatum resulted in a substantial and prolonged impairment in sensorimotor orientation while injection at other sites produced no detectable effects (Dunnett and Iversen, 1982). Both of these types of behavioral deficits have been reported to occur in aged rodents (Bartus *et al.*, 1982; Marshall, 1982). The results of the present investigation, when considered with previous studies, indicate that discrete regional deficits in neostriatal cholinergic function may be related to specific behavioral impairments that accompany old age.

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AGE-RELATED CHANGES IN RAT LIVER PLASMA MEMBRANE PHOSPHOLIPASE A_2 ACTIVITY

DIANA H. PETKOVA,* ALBENA B. MOMCHILOVA and KAMEN S. KOUMANOV

Central Laboratory of Biophysics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Abstract—The alterations of the lipid composition and fluidity of liver plasma membranes as well as the activity of membrane-bound phospholipase A_2 (EC 3.1.1.4) in male and female rats of different ages are investigated. An age-induced increase of the protein/phospholipid and cholesterol/phospholipid ratios is observed, as well as a decrease in the unsaturation index of plasma membrane phosphatidylcholine. These changes induce an augmentation of the steady-state fluorescent anisotropy-r_s and structural order parameter for 1,6-diphenyl-1,3,5-hexatriene-S_{DPH} in plasma membranes. The membrane-bound phospholipase A_2 activity is enhanced with aging, correlating well with the reduced membrane fluidity. The sex-determined alterations in all these parameters is also investigated.

Key Words: aging, membrane fluidity, plasma membrane, phospholipase A_2, phospholipids

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INTRODUCTION

AGING LEADS to considerable changes in the level of cellular lipids and proteins. An age-induced increase of the cholesterol/phospholipid (CH/PL) ratio in kidney and liver (Grinna, 1977; Hagner, 1980) as well as in heart and liver mitochondria (Vorbeck *et al.*, 1982; Lewin and Timiras, 1984) and in adipocyte ghosts (Rifkind *et al.*, 1985) was observed. It is well known that any changes of the protein/phospholipid ratio (Pr/PL) (Vanderkooi, 1974), as well as of the CH/PL ratio (Shinitzky and Lubar, 1974) cause alterations of membrane fluidity.

In adipocyte ghosts, Rifkind *et al.* (1985) have reported that the age-induced elevation of the CH/PL ratio did not lead to an enhancement of the structural lipid order parameter, while Shonga *et al.* (1979) have observed a parallel elevation of the Pr/PL ratio and a decrease of fluidity in in vivo aged erythrocytes. An augmentation of liver plasma membrane microviscosity with aging was reported by Nokubo (1985).

The aging process has been postulated as an accumulation of errors in enzyme activities with time (O'Bryan and Lowenstein, 1974). 5′-Nucleotidase activity in rat liver plasma membranes was significantly elevated in the course of aging (Nokubo, 1985). The specific activities of some membrane-bound hydrolases in rat kidney decrease between 6 and 24 months (Pratz and Corman, 1985). Gaiti *et al.* (1985) have reported a reduced phospholipase activity in different rat brain areas during aging.

*Send correspondence to Dr. Diana H. Petkova.
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Most membrane-bound enzymes strongly depend on their lipid environment (Siekevitz, 1970). Some of them require the presence of definite phospholipids, whereas others depend on the physico-chemical properties of the membrane.

No data are available in the literature concerning the dependence of phospholipase A_2 activity on its lipid environment. In a previous paper (Momchilova *et al.*, 1985) we investigated the influence of different saturated fatty acid diets on rat liver plasma membrane phospholipase A_2 activity. We found that the enzyme was sensitive to changes in the composition and physico-chemical properties of the membrane phospholipids.

The purpose of the following investigations was to follow the relationship between age-induced alterations of phospholipid composition and phospholipase A_2 activity in rat liver plasma membranes.

MATERIALS AND METHODS

Animals

Male and female Wistar strain rats (purchased from the Department of Laboratory Animals, Biological Center, Bulgarian Academy of Sciences) were kept for 16 months in laboratory conditions (in a ventilated room at ambient temperature 22 ± 2°C) and were fed a standard laboratory diet. The animals had free access to food and water. The rats were killed at the age of 3, 8, 11 and 16 months (15 rats per age-group) and their livers were collected. Only rats wth macroscopically normal livers were used for the study.

Isolation of liver plasma membranes

Liver plasma membranes were isolated by procedures described by Wisher and Evans (1975). The purity of the membrane fraction was controlled by the specific activities of the marker enzymes as well as by electron microscopy.

Phospholipase A_2 activity

Membrane-bound phospholipase A_2 activity has been determined by the method of Colard-Torquebiau *et al.* (1975). As substrate 1-acyl-2-[^{14}C] linoleoyl-sn-glycerophosphoethanolamine (Amersham) was used. Incubation medium contained 100 μg plasma membrane protein, 170 nmoles [^{14}C] substrate in 100 mM Tris-HCl, 5mM $CaCl_2$, pH 8.6 in a total volume of 0.2 ml. The released radioactively labeled fatty acids were separated and counted using an LKB-1215 Rackbeta II scintilation counter. The specific activity was calculated as nmoles released fatty acids per min/mg protein.

Analytical methods

Lipids were extracted from the plasma membranes by the method of Folch *et al.* (1957). The phospholipid phosphorus (Kahovkova and Odavic, 1969) and cholesterol (Sperry and Webb, 1950) were determined in the total lipid extract. The individual phospholipids were chromatographed on silica gel 60 thin-layer plates (Merck) in a solvent system containing chloroform/methanol/iso-propanol/0.25% KCl/triethylamine (30:9:25:6:18, by volume) (Touchstone *et al.*, 1980). The protein content of plasma membranes was also estimated (Lowry *et al.*, 1951). The fatty acid composition of phosphatidylcholine (PC) was determined by GLC in a Carlo Erba gas-chromatograph equipped with a flame-ionization

detector isothermally at 190°C and with a 2m column coated with 10% DEGS on Chromosorb $W_{60-80mesh}$ (Pharmacia), with nitrogen as the gas-carrier. The unsaturation index (UI) was calculated as the number of double bonds per molecule of fatty acid × mol%/100.

Fluorescent assays

1,6-Diphenyl-1,3,5-hexatriene (DPH) (Fluka) was used as a fluorescent probe for estimation of plasma membrane fluidity. The steady-state anisotropy of fluorescence (r_s) of DPH was calculated using the following equation:

$$r_s = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 21_{\perp}}$$ (Shinitzky and Barenholz, 1974).

DPH was dissolved in tetrahydrofuran (Merck) at a concentration of 2.0×10^{-3} M. Just before labeling, the solution was diluted 200 fold. DPH was added to the membrane suspension at a ratio of 1 molecule DPH:200 phospholipid molecules. Fluorescence measurements were performed after 2 h incubation at 37°C on a Perkin-Elmer 3000 fluorescence spectrometer.

The lipid order parameter S_{DPH} was calculated by an empirical method described by Van Blitterswijk *et al.* (1981).

Statistical analysis was performed using Student's t-test. Linear regression analysis was also performed.

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RESULTS

The age-induced alterations of the phospholipid composition and the cholesterol level in liver plasma membranes are presented in Table 1. A strong decrease of the phospholipid fractions (compared to 3 month old rats) was observed in both male and female animals. The cholesterol content in plasma membranes of male rats decreased in 8 and 16-month-old rats and was increased in 11-month-old rats. In plasma membranes of female rats the cholesterol content was progressively elevated with aging, with just a slight diminution in 11-month-old rats. The CH/PL ratio in both male and female rat liver plasma membranes was strongly augmented.

Changes in the major phospholipid classes have also been observed. The level of sphingomyelin (SM), PC and phosphatidyl-ethanolamine (PE) gradually decreased in both male and female rats. It is noteworthy, that the SM/PC ratio was reduced in male rats during aging, whereas in female rats it was elevated.

The fatty acid-analysis of PC (results not shown) indicated an age-related decrease of the unsaturated fatty acids, which led to a diminution of the unsaturation index (UI).

Fluidity alterations in plasma membranes isolated from rats of different ages were monitored by the aid of DPH steady-state fluorescent anisotropy (r_s) and the structural lipid order parameter (S_{DPH}) was calculated (Table 2). As shown in the table, r_s as well as S_{DPH} were increased with aging in rats of both sexes, although at a different rate. Plasma membranes of 16-month-old male rats were considerably more rigid than plasma membranes of female rats of the same age.

The age-induced alterations of liver plasma membranes correlated with some changes in

TABLE 1. LIPID ALTERATIONS IN LIVER PLASMA MEMBRANES DURING AGING.[a]

Lipids	Age in Months			
	3	8	11	16
		Males		
Phospholipids	275.60 ± 0.42	170.87 ± 0.29[b]	181.89 ± 0.90[b]	85.46 ± 0.29[b]
Cholesterol	41.34 ± 0.26	33.31 ± 0.18[b]	51.31 ± 0.31[b]	27.77 ± 0.35[b]
CH/PL (mol/mol)	0.300	0.390	0.570	0.650
SM	30.59 ± 0.41	20.77 ± 0.15[b]	13.00 ± 0.41[b]	4.18 ± 0.04[b]
PC	104.17 ± 0.49	75.18 ± 0.34[b]	69.66 ± 0.32[b]	26.73 ± 0.32[b]
PE	30.86 ± 0.44	14.86 ± 0.13[b]	10.00 ± 0.14[b]	3.24 ± 0.17[b]
SM/PC (mol/mol)	0.293	0.276	0.186	0.156
		Females		
Phospholipids	235.86 ± 3.05	188.68 ± 3.40[b]	127.36 ± 1.75[b]	94.34 ± 0.94[b]
Cholesterol	33.02 ± 0.50	38.67 ± 0.21[b]	33.75 ± 0.14[b]	47.17 ± 0.30[b]
CH/PL (mol/mol)	0.280	0.410	0.530	1.000
SM	30.42 ± 1.41	28.67 ± 1.32[b]	17.57 ± 0.76[b]	12.35 ± 0.56[b]
PC	100.00 ± 3.23	90.18 ± 3.96[b]	56.03 ± 1.87[b]	31.60 ± 0.99[b]
PE	20.04 ± 1.65	11.13 ± 0.94[b]	11.08 ± 0.50[b]	6.96 ± 0.56[b]
SM/PC (mol/mol)	0.305	0.318	0.315	0.391

[a]Results are presented as μg/mg protein (Mean ± S.E., n = 15).
[b]$p < 0.001$ as determined by Student's t-test.

the activity of membrane-bound phospholipase A_2. This enzyme was more active in older rats, having a maximal activity in 16-month-old rats. This activity was higher in plasma membranes of male rats (Fig. 1).

The analysis of the relationship between phospholipase A_2 activity and some membranes parameters (Fig. 2) showed a positive linear correlation between enzyme specific activity, the CH/PL ratio and S_{DPH} as well as a negative linear correlation with UI.

DISCUSSION

It is well accepted that aging leads to various changes at the cellular and membrane levels. Our results are no exception in this regard.

TABLE 2. FLUORESCENT ANISOTROPY (r_s) AND STRUCTURAL ORDER PARAMETER FOR DPH (S_{DPH}) IN LIVER PLASMA MEMBRANES FROM RATS OF DIFFERENT AGES [results are mean ± S.E. (n = 10)]

Age in months	r_s		S_{DPH}	
	males	females	males	females
3	0.2250 ± 0.0006	0.2200 ± 0.0009	0.7120 ± 0.0018	0.6990 ± 0.0018
8	0.2300 ± 0.0009[b]	0.2260 ± 0.0012[a]	0.7230 ± 0.0028[a]	0.7130 ± 0.0037[a]
11	0.2390 ± 0.0006[b]	0.2360 ± 0.0012[b]	0.7440 ± 0.0018[b]	0.7380 ± 0.0037[b]
16	0.2690 ± 0.0009[b]	0.2410 ± 0.0018[b]	0.8090 ± 0.0028[b]	0.7480 ± 0.0056[b]

[a]$-p < 0.01$; [b]$-p < 0.001$ (determined by Student's t-test).

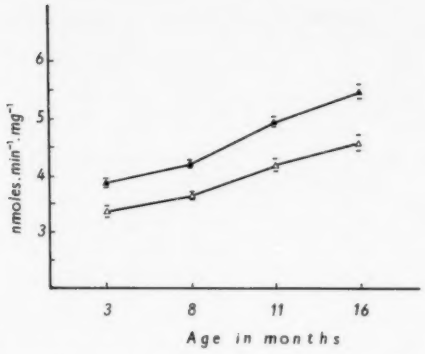

FIG. 1. Activity of phospholipase A_2 in liver plasma membranes of male (▲) and female (△) rats during aging. The enzyme activity was determined with 1-acyl-2-[^{14}C] linoleoylphosphoethanolamine as substrate and was expressed as nmoles released fatty acids per min/mg membrane protein.

We observed significant membrane alterations during aging, which generally cause an increased rigidity of liver plasma membranes.

The total phospholipid content was markedly decreased as a result of the diminution of the major phosopholipid classes. This lead to an augmentation of the Pr/PL ratio. The enhancement of the Pr/PL ratio causes diminution of membrane fluidity (Shinitzky and Barenholz, 1974). The same seems to be true for cholesterol level elevation (Shinitzky and Lubar, 1974). Our results indicate that the CH/PL ratio in liver plasma membranes was increased about 2–3 fold in 16-month-old rats compared to the 3-month ones. This augmentation was more significant in female rats.

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It is very likely that these alterations underlie the increased rigidity in the course of aging, which is expressed by the gradually elevating values of r_s and S_{DPH}. As already mentioned similar results have been reported by Nokubo (1985). The membranes of the older rats possess a more ordered structure of their lipid bilayer also due to the fatty acid composition alterations. This is shown by the decrease in the UI of the membrane PC.

The differences observed between male and female rats of equal ages, are not unexpected, bearing in mind the influence of sex hormones on lipid metabolism. For example, while in immature rats differences in the activities of lipogenic enzymes are almost absent, such differences are apparent in sexually mature animals (Gandemer *et al.*, 1979). Moreover, the differences mentioned above can be reversed by castration.

In a previous paper (Momchilova *et al.*, 1985) we established that the membrane-bound phospholipase A_2 activity in rat liver plasma membranes was strongly influenced by the physical properties of the membranes. Our present investigations are in accord with these results. As already mentioned, phospholipase A_2 was more active in membranes of older rats; its maximal activity being at 16 months of age, i.e. in the most rigid membranes.

The linear correlation between phospholipase A_2 activity and some investigated parameters closely related to membrane fluidity indicates the significance of the membrane physical state for phospholipase A_2 activity. Similar correlations between phospholipase A_2 activity, CH/PL ratio and S_{DPH} have been observed in rat liver plasma and mi-

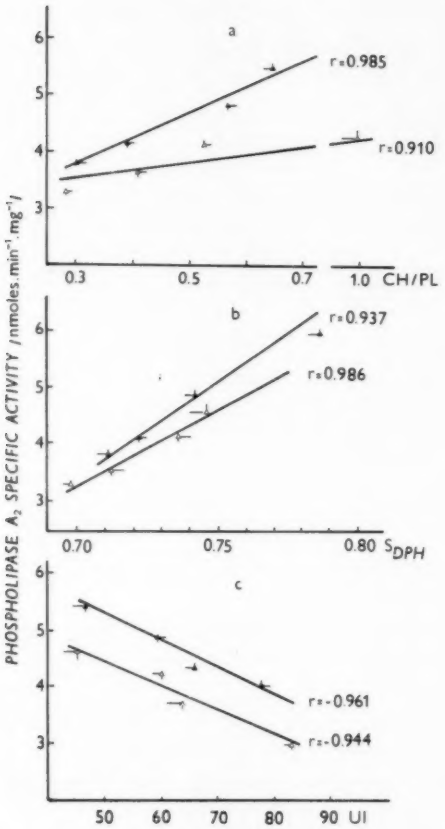

FIG. 2. Relationship between phospholipase A_2 specific activity and S_{DPH} (a), CH/PL ratio (b) and UI (c) in liver plasma membranes of male (▲) and female (△) rats in course of aging. Each point is mean of 15 determinations. The straight lines present the linear regression for all the points. The correlation coeficient (r) was also given.

crosomal membranes treated with exogenous phospholipases or butanol (Momchilova *et al.*, 1986).

Most investigated membrane-bound enzymes are known to prefer a more fluid lipid environment. Liver plasma membrane phospholipase A_2 is an exception in this respect. This enzyme, which is deeply embedded in the lipid bilayer of the plasma membrane obviously requires a more ordered lipid surrounding.

It is very likely that the membrane physical sate is important for regulation of membrane-bound phospholipase A_2, and the age-related changes of the plasma membrane lipid composition might be the reason for enzyme activation.

One should bear in mind, however, that the aging process might be associated with changes in enzyme molecules which could be the reason for the enhanced activity.

Moreover, we suggest that the sex-determined differences in phospholipase A_2 activity

are likely to be due to differences in membrane fluidity of male and female rats rather than to some specific hormonal influence.

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CHRONIC ETHANOL CONSUMPTION AND AGING: CHANGES IN LIPID COMPOSITION OF LIVER MICROSOMES

W. GIBSON WOOD,* LISA S. WILLIAMSON, DAVID ROCCO, and RANDY STRONG

Geriatric Research, Education and Clinical Center, VA Medical Center; and Department of Internal Medicine, St. Louis University School of Medicine, St. Louis, MO 63125

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INTRODUCTION

CHANGES IN lipid composition of liver membranes have been reported to occur both as a consequence of increasing age and as a result of chronic ethanol consumption (Waring *et al.*, 1981; Story *et al.*, 1981; Sutter *et al.*, 1985; Smith *et al.*, 1982). Increases in cholesterol content and total phospholipid content of liver membranes from aged as compared to younger organisms have been reported (Story *et al.*, 1981; Sutter *et al.* 1985). Other age changes include altered distribution of individual phospholipids and an increase in the ratio of saturated to unsaturated fatty acids (Hegner, 1980; Sutter *et al.*, 1985). Chronic ethanol consumption has also been found to affect lipid composition of liver membranes. Cholesterol content has been found to increase in liver membranes of animals consuming ethanol (Smith *et al.*, 1982), and ethanol-induced changes also have been noted in phospholipid content and fatty acid composition (Cunningham *et al.*, 1982; Koivusaari *et al.*, 1981).

If aging and chronic ethanol consumption affect lipid composition of liver membranes, it would be expected that chronic ethanol consumption should have a greater effect on lipid composition of old animals as compared to younger animals. Age differences in response to chronic ethanol consumption have been reported for intoxication, severity of withdrawal, and depolarized release of γ-aminobutyric acid (Wood *et al.*, 1982; Strong and Wood, 1984). This study examined effects of chronic ethanol consumption and increasing age on lipid composition of liver microsomes from 6-month and 28-month C57BL/6NNIA male mice. Liver microsomes were selected because of involvement of the endoplasmic reticulum in lipid synthesis (Siekevitz, 1963), and the microsomal ethanol-oxidizing system, an accessory pathway for ethanol metabolism, is also located in the endoplasmic reticulum (Lieber, 1975).

*Address Correspondence to: W. Gibson Wood, Ph.D., GRECC (111G-JB), VA Medical Center, St. Louis, MO 63125.
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MATERIALS AND METHODS

Animals

Animals used in these experiments were C57BL/6NNIA male mice purchased from the National Institute on Aging colony that is maintained by Charles River Breeding Laboratories, Inc. (Wilmington, MA). Two different age groups, consisting of young (6 months) and old (28 months) mice, were used. These age groups were selected on the basis of previous studies examining chronic ethanol consumption among different age groups of mice (Strong and Wood, 1984; Wood *et al.*, 1982). Animals were maintained in an isolated colony room with temperature and humidity automatically regulated.

Materials

All chemicals used for lipid composition assays were obtained from Sigma Chemical Company (St. Louis, MO). Ethanol (95% w/v, U.S. Industrial Chemicals Company, New York, NY) was administered in the Bio-Serv liquid mouse diet (Bio-Serv, Frenchtown, NJ). Control animals received the Bio-Serv liquid mouse control diet.

Ethanol administration

Ethanol was administered in the Bio-Serv liquid mouse diet (Bio-Serv Inc., Frenchtown, NJ) at a final concentration of 5% (w/v). Procedures for diet administration have been described previously (Wood *et al.*, 1982). Briefly, the diets were administered in 50 ml tubes. Liquid diet consumption was measured at 3:00 p.m. each day and fresh diets administered. Age-matched, pair-fed control animals were administered the Bio-Serv control liquid diet that was based on the amount consumed by the ethanol animals the previous day. The Bio-Serv ethanol and control diets are casein-based liquid diets. Maltose-dextrins were isocalorically substituted for ethanol in the control diet. Water was available *ad libitum* for all animals. Diets were administered for 24 days. Diets were removed from the ethanol and control groups on Day 25, and animals were killed between 8:00 a.m. and 10:00 a.m. that same day.

Membrane preparation

Liver was weighed and homogenized in 10 volumes of ice-cold 0.32 M sucrose using a Teflon glass homogenizer. Liver microsomal membranes were isolated by differential centrifugation using procedures described previously (Armbrecht *et al.*, 1983; Trotta *et al.*, 1975). Protein content of liver microsomes was measured by the method of Lowry *et al.* (1951). Microsomal purification was monitored using glucose-6-phosphatase as a marker enzyme (Duttera *et al.*, 1968) and did not vary significantly with age. Liver microsomes were prepared separately for each animal, and all assays were performed in triplicate per animal.

Lipid analysis

Liver microsomes were extracted with 10 volumes of chloroform:methanol, 2:1 (v/v), according to the procedure of Folch *et al.* (1957). After phase separation, the extracted lipids were taken to dryness with nitrogen, resuspended in 3 ml of chloroform, and stored at −70°C until assayed. Aliquots of the lipid extract were assayed for total phospholipid

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and cholesterol content (Bartlett, 1959; Bowman and Wolf, 1962). Major phospholipids of the lipid extract were separated by two-dimensional, thin-layer chromatography on silica gel G based in part on the method of Horrocks and Sun (1972). This method results in approximately 95% recovery of phospholipid (Sutter *et al.*, 1985; Wood *et al.*, 1984). The solvents used were chloroform/methanol/ammonium hydroxide (135/55/10 ml) and chloroform/methanol/acetone/ammonium acetate/glacial acetic acid (140/60/55/10/35 ml) for the first and second dimensions. After development in the first dimension, the plate was dried briefly and exposed to concentrated HCL fumes for 3 min to cleave the alkenylether bond (Horrocks and Sun, 1972). The separated phospholipids were visualized by brief exposure to iodine vapor and scraped from the plate. Assays of individual phospholipids were done by determination of phospholipid phosphorus using the method described above for total phospholipids. The separated phospholipids were quantitated as μg/mg protein and expressed as percent of total phospholipid.

Statistics

All results were expressed as the mean ± S.E.M. of the number of animals per group. Liver microsomes were prepared separately for each animal, and all assays were performed in triplicate per animal. Statistical comparisons were made using analysis of variance (Kirk, 1968) and Student's *t*-test (Dixon and Massey, 1969). Individual comparisons were made after a significant analysis of variance.

RESULTS

Body weights, when animals were sacrificed on Day 25, did not differ significantly among the four groups (Table 1). However, changes in body weight between Day 1 and Day 25 differed with age. Each of the four groups showed an increase in body weight. This increase was significant ($p < .01$) for all of the groups except the old control group. Ethanol consumption (mean ± S.E.M.) averaged over 24 days for the young and old ethanol groups, was 25.55 ± .54 and 25.28 ± 1.12 grams of ethanol per kilogram of body weight, and was not significantly different.

Liver weight was affected by both age and ethanol consumption (Table 2). Overall, livers of aged animals were heavier as compared to younger animals ($p < .001$). Ethanol

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TABLE 1. BODY WEIGHTS

Treatment	Age (months)	Body Weight (grams) Day 1	Day 25
Ethanol diet	6	28.81 ± 0.63*	32.66 ± 0.67
Control diet	6	28.44 ± 0.51*	31.68 ± 0.50
Ethanol diet	28	29.63 ± 0.99*	32.51 ± 1.32
Control diet	28	29.86 ± 0.80	31.30 ± 0.72

Animals were weighed on the first day and last day of the experiment. Paired comparisons were made between Day 1 and Day 25. Data are the means ± S.E.M. of 8 animals per group.

*$p < .01$

TABLE 2. LIVER WEIGHTS AND LIVER WEIGHT TO BODY WEIGHT RATIOS

Treatment	Age (month)	Liver Weight (grams)	Liver Weight/Body Weight
Ethanol diet	6	1.440 ± .039	.0442 ± .0007
Control diet	6	1.218 ± .034*	.0383 ± .0011*
Ethanol diet	28	1.767 ± .144	.0536 ± .0036
Control diet	28	1.535 ± .069	.0495 ± .0022

*$p < .01$ as compared to all other groups.
Values are the means ± S.E.M. of 8 mice per group.

consumption also had a significant effect on liver weight ($p < .008$). Ethanol consumption resulted in a significant increase in liver weight for both the young and old ethanol groups. Both aging and ethanol consumption significantly increased the ratio of liver weight to body weight ($p < .03$). The young control animals had the significantly lowest liver weight and liver-to-body weight ratio ($p < .01$) as compared to the other groups.

Ethanol consumption affected phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Table 3 shows that the percentage of PC was significantly lower ($p < .004$) and the percentage of PE was significantly higher ($p < .003$) in microsomes of ethanol-treated animals when compared to microsomes of control animals. Age differences were not found for any of the individual phospholipids.

Cholesterol content and total phospholipid content did not differ significantly as a result of ethanol consumption or increasing age (Table 4). However, the cholesterol-to-phospholipid ratio of the ethanol groups was significantly lower ($p < .009$) when compared to the control groups.

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TABLE 3. PHOSPHOLIPID CONTENT IN LIVER MICROSOMES OF DIFFERENT TREATMENT AND AGE GROUPS

	Treatment/Age			
	Ethanol		Control	
Lipid*	6 months	28 months	6 months	28 months
	% Distribution			
PC	54.91 ± .92**	54.90 ± .86**	57.76 ± 1.33	57.77 ± .91
PE	22.61 ± .43**	23.38 ± 1.24**	18.97 ± 1.06	20.08 ± 1.00
LPE	.39 ± .03	.25 ± .02	.30 ± .03	.31 ± .03
PS	3.59 ± .19	3.43 ± .17	3.52 ± .17	3.59 ± .34
PI	11.61 ± .64	11.19 ± .26	10.59 ± .46	10.75 ± .32
SP	3.79 ± .27	3.54 ± .24	3.64 ± .41	3.59 ± .27
Cl	2.04 ± .41	1.74 ± .17	2.65 ± .41	2.10 ± .45

*PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SP, sphingomyelin; Cl, cardiolipin.
**$p < .004$ as compared to young and old control groups.
Data are expressed as mean ± S.E.M. of 8 animals per group.

TABLE 4. CHOLESTEROL AND TOTAL PHOSPHOLIPID CONTENT OF LIVER MICROSOMES

Treatment	Age (months)	Cholesterol (μg/mg protein)	Phospholipid (μgP/mg protein)	Cholesterol/ Phospholipid (molar ratio)
Ethanol diet	6	57.50 ± 3.85	348.58 ± 13.91	.2659 ± .0154*
Control diet	6	63.29 ± 5.53	317.13 ± 19.75	.3221 ± .0204
Ethanol diet	28	58.81 ± 5.08	323.18 ± 11.08	.2929 ± .0228*
Control diet	28	65.82 ± 5.68	293.75 ± 9.47	.3605 ± .0278

*$p < .009$ as compared to the young and old control groups.
Data are expressed as mean ± S.E.M. of 8 animals per group.

DISCUSSION

Both chronic ethanol consumption and increasing age were found to have specific effects on liver. However, ethanol-induced changes in liver did not differ between the young and old groups. These results indicate that aged animals are able to adapt to effects of chronic ethanol consumption with respect to the parameters measured in the present study. This capacity to adapt has not been observed for intoxication, severity of withdrawal or development of cellular tolerance in response to chronic ethanol consumption in old as compared to younger mice where ethanol consumption was for 14 days and 28 days, respectively (Wood *et al.*, 1982; Strong and Wood, 1984). These age differences were mediated by effects of ethanol on brain function, suggesting that age differences in effects of chronic ethanol consumption may be organ specific.

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Liver weights were highest in young and old ethanol animals and old control animals as compared to young control animals, but did not differ significantly between the two ethanol groups. A possible explanation for the increase in liver weight found with chronic ethanol consumption and aging may have resulted from an increase in triglycerides that occur with chronic ethanol consumption and increasing age (Kritchevsky, 1980; Lieber, 1975). It also has been reported that protein content of liver increases as a result of chronic ethanol consumption (Lieber, 1975).

Chronic ethanol consumption had a similar effect on lipid composition of young and old ethanol groups. The percentage of PC declined and the percentage of PE increased in microsomes of young and old ethanol mice. These changes in individual phospholipids along with increased liver weights are characteristic of choline deficiency (Pelech and Vance, 1984). However, such an explanation for the phospholipid changes in the ethanol groups would not be expected given the similar compositions of the liquid diets for the ethanol and control groups and the utilization of pair-feeding procedures. An alternative explanation is that specific enzymes involved in the biosynthesis of PC via the Kennedy pathway are inhibited by ethanol, e.g. choline kinase, cytidylytransferase, and choline-phosphotransferase. Animals fed an ethanol diet for two weeks have been shown to have a decrease in the biosynthesis of PC via the Kennedy pathway (Skurdal and Cornatzer, 1972).

Cholesterol content did not differ as a result of 24 days of ethanol consumption or between the two different age groups. Ethanol-induced increases in cholesterol content of liver membranes have not been reported consistently (Koivusaari *et al.*, 1981; Hirayama *et*

al., 1979). Recently, a reduction in cholesterol content of brain synaptic plasma membranes in ethanol-tolerant animals were reported (Harris *et al.*, 1984). Differences in cholesterol content also were not found between young and old animals. Increased cholesterol content has been reported in rats (Van Bezooijen, 1984) and primates (Sutter *et al.*, 1985), and no differences in cholesterol content of liver plasma membranes of young and old rats (Hegner, 1980). Strain and species differences may influence cholesterol content in addition to such factors as diet and physical activity (Kritchevsky, 1980). Total phospholipid content also did not differ as a result of chronic ethanol consumption or age. Like cholesterol content, it has not been consistently shown that chronic ethanol consumption affects total phospholipid content (Smith, 1982). Effects of ethanol on total phospholipid of liver microsomes have included increased content (Koivusaari *et al.*, 1981), no effect (Cunningham *et al.*, 1982), and a decrease in content (Miceli and Ferrell, 1973).

Phospholipid distribution did not differ with age. We previously reported (Sutter *et al.*, 1985) an increase in PC of liver microsomes from different age groups of non-human primates (Macaca Nemestrina). In a recent review (Van Bezooijen, 1984), it was concluded that no consistent age changes in distribution of phospholipids of liver microsomes from mice and rats have been found.

Animals 6 months and 28 months of age were used in this study. It is possible that effects of ethanol consumption on liver might differ in an intermediate age group (e.g. 12–16 mo). We have found previously, however, that in vitro effects of ethanol on membrane fluidity and neurotransmitter release were similar between animals 12 months and 24 months of age (Armbrecht *et al.*, 1983) and 14 and 28 months (Strong and Wood, 1984).

The liver of aged animals showed the same capacity to respond to chronic ethanol consumption as compared to younger animals. This capacity does not extend to effects of ethanol on brain function, but is consistent with the finding that metabolism of ethanol does not differ with age (Vestal *et al.*, 1977). Other liver membranes, e.g. liver plasma membranes and mitochondrial membranes, should be examined in order to determine if effects of chronic ethanol consumption in different age groups might occur in other liver organelles.

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SUMMARY

Both aging and chronic ethanol consumption have been found to produce changes in lipid composition. Severity of intoxication, withdrawal and release of γ-aminobutyric acid following chronic ethanol consumption have been shown to be associated with age. It was predicted in this study that aged mice would differ in response to ethanol-induced changes in lipid composition of liver microsomes as compared to younger mice. Two different age groups of C57BL/6NNIA male mice (6 and 28 months) were administered an ethanol or control liquid diet for 24 days. Liver microsomes were prepared on Day 25. Age and ethanol consumption significantly affected liver weight and the ratio of liver weight to body weight. PC significantly decreased and PE significantly increased in both the young and old ethanol groups. Cholesterol and total phospholipid content were not affected by age or chronic ethanol consumption. Aged animals were able to adapt to the effects of chronic ethanol administration to the same extent as younger animals. These findings differ from studies that have examined effects of chronic ethanol consumption on behavior and neuro-

transmitter release among different age groups of mice. While the results are specific for liver microsomes, it appears that chronic ethanol consumption has less of an effect on liver function as compared to brain function in aged mice.

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MEETING REPORT

Highlights of the Minutes of Meetings of the Council and Executive Committee of the International Association of Gerontology (IAG) July 14–16, 1985 and April 5–6, 1986

DELIBERATIONS AND ACTIONS OF IAG COUNCIL

On July 14 and 16, 1985, at the Congress in New York City, USA, President E. W. Busse, certifying a quorum of 50 members of Council present, opened the meeting with the announcement of an active program to develop gerontology internationally.

Treasurer's Report

IAG Treasurer Schmitz-Scherzer reported a current fund balance of US $45,017.

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Election of Officers

On the nomination of President Busse, Council unanimously elected George L. Maddox (USA) Secretary General and Vice-President of the Association and Leonard Hayflick (USA), Treasurer.

Committee on By-laws Report

Simon Bergman (Israel, European Region) reported for the By-laws Committee (G. Andrews, Australia/Asia Oceania Region; J. Gonzalez-Aragon, Mexico/Latin American Region; E. Brody, US/North American Region; and G. Maddox, US/Committee Secretary). He noted a substantial review led to recommendations to edit and re-order some items for purposes of clarity and in a few instances recommendations for substantive changes. On March 15, 1985, all constituent societies of the association received a copy of the current By-laws, a copy of the proposed revisions, and a memorandum identifying significant changes proposed to clarify terms of office, regional responsibilities, and conditions of membership in the Association.

Council, after appropriate discussion on July 14, 1985, accepted in principle the Revised By-laws as proposed, asked that the document receive one final legal review, and instructed the Secretary General to translate into French and file the Revised By-laws in Belgium, where the original 1950 By-laws of the association are registered.

With the assistance of Member of Council, W.J. Dekoninck of Belgium, the Revised By-laws are expected to be appropriately filed in Belgium by the end of 1986.

UN Committee on Terms and Definitions

Hans Thomae (West Germany), who represented IAG on this committee, reviewed an initial meeting and recommended that the IAG pursue the matter on its own as well as in cooperation with the UN. This recommendation was approved. (Further action was taken on this matter at the meeting of the Executive Committee. See below).

Regional Reports

Reports were heard from A. Viidik (Denmark/Europe); G. Andrews (Australia/Asia-Oceania); J. Gonzalez-Aragon (Mexico/Latin America); and J. Tobin (US/North America). Regional meetings in 1987 were announced for Brighton, UK, and Bangkok, Thailand.

XIVth International Congress, Mexico, 1989

President-elect S. Bravo Williams announced that J. Gonzalez-Aragon would be the Congress Vice President; P. Babb, Secretary; and E. Contreras, Treasurer. The exact time and location of the Congress had not been determined (see below).

International Congress, 1993

E. Beregi extended an official invitation to Budapest, Hungary. Argentina, Australia, and Spain expressed interest in being considered.

Sandoz Prize

President Busse appointed F. Anderson (UK), E. Beregi (Hungary) and H. Orimo (Japan) to review procedures for awarding the Sandoz Prize. The committee report which was accepted by Council on July 16, 1985, and subsequently by Sandoz management, recommended the continued use of the IAG Executive Committee as the selection committee but augmented as required to be representative of the diverse disciplines in gerontology. M. Bergener will continue as coordinator but will not have a vote in the final selection.

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Dues and Budgeting

Treasurer Hayflick reviewed the complex procedures required to secure payment of annual dues and his plans to contact constituent societies.

J. Brocklehurst requested, and Council seconded, a request that a working budget for IAG be prepared and distributed to Council.

Prospective Members

The Latin American Region recommended for membership Costa Rica, Paraguay, Cuba, Ecuador and several individuals. Formal applications were awaited.

The full minutes were circulated to members of the Executive Committee for comment and amendment as required on November 5, 1985.

DELIBERATIONS AND ACTIONS OF THE EXECUTIVE COMMITTEE

Treasurer's Report

Treasurer Hayflick's report of a fund balance of US $45,284.45 on March 5, 1986, was approved.

Committee on Terminology

The Executive Committee encouraged H. Thomae to pursue this project for IAG initially begun with the UN and to use the Executive Committee as appropriate. US $200 was approved for his use as needed.

Developing Nations Network

G. Andrews (Australia) has proposed, following a recommendation at the 1985 Congress, to continue The Developing Nations Network. The Executive Committee approved this idea in principle and agreed to appropriate financial support pending a formal budgetary request.

Allocation of Funds to Regions

The Executive Committee approved US $100 for each region to assist in inter-regional communication plus an additional US $200 on request for the promotion of IAG objectives. An additional allocation of $300 per region will be considered in 18 months.

IAG Newsletter

Highlights of minutes of meetings of Council and the Executive Committee will be published in journals in the various regions. Additionally, up to US $1,000 was authorized to the Secretary-General to develop an initial issue of an IAG newsletter.

New Members of the Association

Acting in behalf of Council after reviewing documents as required by the By-laws, the Executive Committee approved the membership of gerontological societies in Costa Rica, Paraguay, and Singapore contingent on payment of dues. The individual memberships of D. Dutari-Estevez (Panama) and A. Guevara D'Arriello (Ecuador) are contingent on the payment of US $25 in annual dues.

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Update on XIVth IAG Congress, 1989

The next Congress will be held in Acapulco "probably in the last week of June or the first week of July, 1989." A Congress logo was approved. The Mexican Organizing Committee is now mobilizing the Congress Scientific Planning Committee as required by By-laws and is contacting regional leadership for suggestions regarding the program.

The Executive Committee discussed with President-elect S. Bravo Williams the financial responsibilities of the Mexican Organizing Committee for the Congress and the need for an estimated budget for the Congress to be reviewed by the planning committee. Memoranda of agreement regarding these matters were to be received by the Executive Committee by July, 1986.

Next Meeting of the Executive Committee

The next meeting is scheduled in conjunction with the regional meeting in Brighton, UK, September, 1987.

The full minutes of the Executive Committee were sent to its members on April 28, 1986.

George L. Maddox, Ph.D.
Secretary General and
Vice-President, IAG

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ANNOUNCEMENT

1987 Grant Program for Biomedical Research in Gerontology and Geriatrics

The American Federation for Aging Research is offering one-year grants, up to $25,000 each, in support of clinical and basic research on all biomedical aspects of aging. Between 30 and 40 grants will be made. AFAR is especially interested in proposals dealing with incontinence, memory loss and confusion, and sensory failure. Proposals from investigators entering the field of aging are especially welcome.

AFAR is also offering the Anna A. Greenwall Award in Basic Research in Gerontology and Geriatrics. The Greenwall Award provides $50,000 annually for a three-year period, subject to annual review of progress, and may be used for salary, equipment, supplies and other research costs, but not for overhead or indirect costs. Applicants for the Greenwall Award must not have attained their 35th birthday by July 1, 1987.

Applicants for the general AFAR grants and for the Greenwall Award must be citizens or permanent residents of the United States and must be affiliated with an institution in the United States where the research will be conducted. Applications must be postmarked no later than January 15, 1987, and funding will begin on July 1, 1987.

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Where to apply: Information and applications may be obtained from:

Harold Epstein
Executive Vice-President
American Federation for Aging Research
335 Madison Avenue, 4th Floor
New York, New York 10017
212/503-7600

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Waneen Spirduso
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IN CELEBRATION OF
THE EIGHTIETH BIRTHDAY
OF
NATHAN W. SHOCK, Ph.D.

25 December 1986

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PREFACE

THIS double issue of EXPERIMENTAL GERONTOLOGY was assembled as an 80th birthday tribute by some former and present colleagues of a great man,—Dr. Nathan W. Shock.

We have assiduously avoided calling this volume a "Festschrift," having in mind the aging and eminent physicist who was fearful of the approaching threat. In an article entitled "Fest me no Schriften," he said that he "did not wish to become a pretext for the unloading of unrefereed and possibly otherwise unpublishable contributions by former students and colleagues." It is said that the Duke of Wellington once declined a dedication, explaining that he had been obliged to make a rule to do so "because in his situation as Chancellor of the University of Oxford, he had been much exposed to authors."

We can, at the least, assure Nathan that all of the scientific contributions in this issue were peer reviewed and that the primary calling of most of the contributors is "scientist" and not "author."

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There is no individual who has contributed more to the present recognition of gerontology as a scientific discipline in the United States and, perhaps the rest of the world, than has Nathan Shock. Those who achieve such eminence are, in their later years, often referred to as the "Father" of that field. Nathan has learned to endure that appellation for at least the last decade. In this volume some of the scientific children and grandchildren of Nathan Shock have assembled to pay homage to their mentor.

But there is one extraordinary exception to this statement.

Dr. A. Baird Hastings, who was, himself, the scientific mentor of Nathan Shock, and is now 90 years old, introduces this collection with a touching tribute to his former student. He may properly be called the "Grandfather" of Gerontology.

The substantive scientific contributions to gerontology by Nathan Shock have been profound, but he is equally recognized for his early insistence that the rigorous scientific standards common to other fields of biology be applied equally to gerontology. The field had long suffered from slipshod data acquisition compounded by loose interpretation. Nathan Shock, more than any other individual, insisted that the scientific method be rigorously applied to gerontological research. As a result of this, his laboratories at the Gerontology Research Center in Baltimore, Maryland, spawned a cadre of gerontologists who, themselves, have become leaders in this now burgeoning field.

Today's gerontologists are indebted to Nathan Shock for more than his scientific achievements. His resolute determination and courage to promote gerontology as a legitimate field for scientific investigation was made in an atmosphere of almost total neglect by the scientific establishment and even some hostility by the bureaucracy. Like most advocates of unpopular causes he met with the action, or more precisely, the inaction of an assortment of naysayers. Nevertheless Nathan remained firm in his committment and ultimately

triumphed to the extent that, today, his efforts have propelled Gerontology into a vital and respected scientific discipline.

Nathan Shock was one of the first to appreciate the value of longitudinal studies in gerontology; an insight that has contributed enormously to our understanding of the normal aging process. He established the Baltimore Longitudinal Study of Aging in 1958 which now includes about 650 male and 350 female participants. The results from some of these studies have shattered some of our most cherished beliefs. Several of these are described in the definitive history of this study whose senior author is Nathan Shock and is published under the title "Normal Human Aging: The Baltimore Longitudinal Study of Aging" (U.S. Government Printing Office, Washington, D.C., NIH Publication NO. 84-2450, 1984). This, incidentally, is the 295th publication in a list of 300 papers published by Nathan.

Nathan earned his undergraduate degree in chemistry at Purdue and a Ph.D. in Physiological Psychology at the University of Chicago in 1930. After a two-year post as Research Associate and Instructor at the University of Chicago he spent nine years at the University of California, Berkeley, first as a Research Associate and then as an Assistant Professor.

From 1941 to 1977, Nathan Shock served as Chief of the Gerontology Research Center. After the establishment of the National Institute on Aging in 1975 Nathan became its first Scientific Director. He retired in 1976.

Among his many awards and honors are a D. Hum. Lett. (Hon.) from Johns Hopkins University; Fellow of the American Psychological Association; President and Fellow of the Gerontological Society of America; Modern Medicine Award; Ciba Foundation Award; Brookdale and Kleemeier Awards from the Gerontological Society of America; Willard O. Thomson Award from the American Geriatrics Society; Superior Service Award from the U.S. Dept. of HEW; Ollie A. Randall Award from the National Council on Aging; Kesten Lectureship, University of Southern California; Distinguished Achievement Award from the American College of Nutrition; First Award of Distinction from the American Federation for Aging Research and President of the International Association of Gerontology.

Today Nathan Shock is Scientist Emeritus of the National Institutes of Health. He continues to be active in research, writing, professional society activities and celebrating birthdays. We are proud to dedicate this collection of papers to Nathan Shock on the occasion of his 80th birthday when, like the other fortunate among us, he experiences the same phenomenon to which he has dedicated a life of scientific interest.

LEONARD HAYFLICK, PH.D.
Professor
Center for Gerontological Studies
University of Florida
Gainesville, Florida

RICHARD C. GREULICH, PH.D.
Director
Gerontology Research Center
National Institute on Aging
Baltimore, Maryland

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LETTER

Nathan Shock, Ph.D.
Scientist Emeritus
National Institute on Aging
Gerontology Research Center
Baltimore, Maryland 21224

Dear Nathan:

Nothing would please me more than to be able to contribute an article to the forthcoming issue of Experimental Gerontology, honoring Dr. Nathan Shock's 80th birthday.

As you know, I have just survived my 90th birthday, and I have long ago abandoned everything *Experimental*. All I have left are some vivid memories.

One of these is of a graduate candidate, referred to me by Professor Thurston (Chicago psychologist). I was in the Physiology Department to the, then, medical school. We, Thurston and I, had agreed that if a likely student showed up, he would let me make a physiologist of him, then he would give him psychological training. Nathan W. Shock from Purdue seemed to be such a candidate. He rose to the occasion magnificently. First he devised a micro-pipette which lets one get all necessary data on 0.1 ml of whole blood. The data consisted of: (1) a hematocrit reading; (2) a plasma colorimeter pH reading; and, finally, a total blood CO_2 concentration. These data applied to a nomogram (devised by Shock) and gave the pCO_2 and buffer base which led to the Acid-Base balance charts used in the next two papers. The first was on variations encountered in normal males and females, the second on Acid-Base pathways followed back to normal after displacement by NH_4Cl, $NaHCO_3$, CO_2 deficit and CO_2 accumulation.

The work was elegantly done and, as far as I know, has not been surpassed. The four papers published in the Journal of Biological Chemistry comprised the bulk of his Ph.D. thesis.

After a short post-doctoral stay at the University of Chicago, he found himself the physiologist of a group following 50 boys and 50 girls through puberty at Berkeley, California. The study continued for eight years, after which he became (in 1941) Chief of the Gerontology Research Center of the U.S. Public Health Service, located at the Baltimore General Hospital. It now has its own separate building for research, over which he presided effectively until his retirement.

Such has been the birth, maturation and condensed career of Mr. Gerontologist Number One!

Never did I have a better, more successful student. When I would suggest duplicate

analyses, they would usually appear in triplicate. That describes Nathan Shock and his search for accuracy in Gerontology.

Nathan, I salute you on your 80th anniversary. I only wish I could be present on this well-earned occasion.

With pride and affection,

A. Baird Hastings, Ph.D.
Scripps Institution of Oceanography
Physiological Research Laboratory
University of California, San Diego
La Jolla, CA 92093

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LETTER

Nathan Shock, Ph.D.
Scientist Emeritus
National Institute on Aging
Gerontology Research Center
Baltimore, Maryland 21224

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Dear Nathan:

Congratulations to you on your 80th birthday and for your parental role in the birth of gerontology. I would like to have contributed an article to *Experimental Gerontology* on the occasion of your birthday but events of this year prevented me from sitting down and thinking through either a theoretical piece or organizing an empirical article.

Some of us had the opportunity of watching the growth of gerontology and have had the pleasure of seeing you serve both as an instigator of the birth and as a watchful parent of the growth of gerontology. I was at the Gerontology Center in Baltimore when you went to the organizing committee of the IAG in Liege, Belgium in, I believe, 1948. Subsequently, this resulted in the formal organization and then the subsequent congresses of gerontology, although the meeting in St. Louis in 1951 is described as the second meeting of the International Association of Gerontology. During those years you were serving as secretary to the Josiah Macy, Jr. Foundation and produced the published reports that give some insight into the foundation of gerontology in the U.S.

I remember the fact that you came to Baltimore from Berkeley and arrived in Baltimore on Pearl Harbor Day, December 7, 1941 to start what developed into the Gerontology Research Center. What a time to arrive in a new city and take up a new job when war time preoccupations were a long way from the issues of gerontology. Immediately after World War II, you began to assemble the staff that has now led to the preeminent Gerontology Research Center in Baltimore. There were several qualities to your perspective that I think have been extremely important in your influence on the development of gerontology. One of these has been a multidisciplinary orientation to the basic facts of the field. It almost appears at times as though the phenomena of aging become the property of disciplines. Lost in the preoccupations of the disciplines is the fact that complex organisms show aging and that sometimes our perspectives are distorted because of disciplinary preoccupations. Aging is not a product of the disciplines, it is a naturally occurring set of phenomena about which we gain some insight from the different disciplinary perspectives.

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It seems to me your orientation as a scientist grew out of the work that you did with both a psychologist at the University of Chicago, Professor Louis Thurstone, and the Chairman of Biological Chemistry, Baird Hastings. It was that experience which was later transported to Berkeley where you jointly looked at the development of children from a physiological and psychological perspective. How rare your broad perspective is still at this time. Gerontology, through your guidance, has remained a broadly oriented field with resistances to fadism.

Another contribution you had made to gerontology has been to insist on solid empirical grounding. You always placed high value on empirical research. You and your colleagues in Baltimore have made major contributions to empirical research that have provided the basis for major progress in the field. The field of gerontology is now sufficiently well empirically grounded that I think we are now ready for new interest in theory and ideas that will link together various phenomena of aging. It has been through your efforts that we have arrived at this stage in history where we more clearly realize the potential of gerontology for human well being. This was only a remote promise at the onset of your career in the field 45 years ago.

You invited me to consider a career in gerontology in 1946 and I moved to Baltimore in 1947. Thirty nine years later, I am still impressed by the soundness of my decision to join you in Baltimore and to share the prospects for research in gerontology.

There is one other point that I would make about you and your career that grew out of a talk I heard you give in San Francisco a few years ago. I noted the major ideas that you introduced and I was impressed by the fact that not one of the major concepts on which your talk was based could have been referenced at the onset of your career. What this means is you grew with your field and you were using concepts none of which were discussed at the time you were a graduate student. This, it seems to me, is one of the most important aspects of a career in science, that is, one can keep up with the field if you are so motivated. You were so motivated and, not only were you there at the birth of gerontology, but the field grew with you. I think you have provided all of us as well as the field of gerontology with a model of growing up, if not growing old.

James E. Birren, Ph.D.
Dean
Ethel Percy Andrus Gerontology Center
University of Southern California
Los Angeles, CA 90089

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GERONTOLOGY COMES OF AGE– DR. SHOCK'S 40-YEAR LEGACY

MARVIN J. YIENGST (AS TOLD TO DANIEL S. ROGERS)

Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, Maryland

About the Author

Marvin J. Yiengst retired from the Gerontology Research Center as a Research Chemist in 1972. He was the first employee on site at the original Gerontology Unit being hired as a medical technician in 1940. His reminiscences of the early years of gerontology with Dr. Shock offer valuable lessons to those who, at times, might become discouraged with the slow progress in aging research or the budgetary hardships scientists frequently face. Marvin, since his retirement, has become a winemaker *par excellence*, winning numerous regional and national awards for his Chardonnays and his blend of Seyval Blanc and raspberries. The latter won him second prize at the 1978 American Winemakers Society (AWS) national conference. He was named the Society's Outstanding Member of the AWS national conference in 1985. His winemaking tips appear frequently in "The Artful Winemaker" column of the AWS Journal.

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IN 1940, THE U.S. Public Health Service was approached and asked if they would support a section concerned with gerontology. They agreed to finance this undertaking to some extent by paying for a part-time secretary and a technical assistant to a director. The salary of the director, Edward J. Stieglitz, was paid by the Josiah Macy, Jr. Foundation in the form of a one year endowment, after which it was presumed the Public Health Service would pick up his salary.

I was hired by Dr. Stieglitz and my duties were assigned by him. I was told to report to the Baltimore City Hospitals, whereas Dr. Stieglitz was stationed in Bethesda, Maryland, at the main campus of the then National Institute of Health (NIH). Baltimore City Hospitals was selected as the site for the unit because it had an unusually large population of elderly, indigent males, housed in an infirmary. This group numbered about 800 individuals and was considered as a potential source of subject material.

My assigned duties consisted of conducting research in the fields of renal function, as outlined by the director. By October of 1941, it became evident that the then acting director

Correspondence to: Daniel S. Rogers, Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224.
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would not continue in his position in the Public Health Service. The search for a new director commenced.

October of 1941 was a beautiful, balmy, springlike month in Baltimore. I remember one particularly fine morning when a zephyr came in from the West Coast. This was a land-based zephyr, namely a Lincoln Zephyr. It carried four occupants, namely Dr. Nathan W. Shock, the newly appointed unit director, his wife Margaret, and their two sons.

Dr. Shock had some very good ideas on the promising aspects of aging research. One of the things that was eventually pursued involved further studies on renal function. Also, acid-base balance and similar areas.

We were attached to the Division of Physiology of the NIH for administrative and budget purposes. Because of the fact that budgets were quite limited in those days some of our favorite laboratory tools consisted of hacksaws, handsaws, and a hatchet.

In many cases, it became necessary to fabricate our own equipment. This was one means of accomplishing the job. Originally, I was assigned to a small ward laboratory which was designed for conducting the routine blood and urine analyses needed by the ward resident. The room was very small and was fine for my purposes. However, when Dr. Shock came on the job, we secured more equipment. Some of it came from California where he had been stationed previously. It was customary to put all the equipment and other laboratory ware on wheels. The duty of the first person in each morning, either Dr. Shock or myself, was to wheel out into the hall this equipment so we could enter the lab.

The fourth floor where we were stationed, was a chronic ward for indigent females. Many of these ladies were in wheelchairs and our equipment certainly did add to hall congestion and made us rather unpopular, not only with the City Hospitals' officials but also with the fire department. Consequently, it was decided we needed more room to pursue our studies. An old dormitory in the infirmary was presented as a first choice. It was a huge room that had been abandoned as a dormitory because it had no fire escape. In years gone by it had been a bakery. There was only one entrance to it and there was a huge chimney at the far end which served as a gathering place for pigeons. They were finally discouraged from using the chimney after we used it as a hood when carrying out Kjeldahl digestions. This procedure liberated sulfuric acid fumes which proved unacceptable for the resident fowls.

The building was quite old. It was on the north side of the hospitals' grounds. The windows were very loose and rickety. The heating was completely inadequate in winter and we were not too distant from the Pennsylvania Railroad marshalling yards. As a result, we were constantly bombarded with soft coal dust and dirt. All the equipment and instruments had to be carefully covered daily. It became the duty of the first person coming on the scene each day to sweep, dust and generally clean up the working space. In winter the first to arrive would also light the gas heat water baths to warm up the area. Usually, by mid or late morning, the place became more acceptable.

One interesting sidelight to this is when Dr. Shock, on one of his trips to the Bethesda stores, had a hard time convincing the personnel there of the need for tremendously oversized laboratory coats, since both of us were rather slender fellows. They finally agreed to issue the coats when it was explained to them that these coats had to be worn over overcoats because of the inadequate heating in our laboratory. In the summertime, of course, the opposite held and we were subject to the vagaries and extremes of the Baltimore weather.

Several years prior to this time, some rather sophisticated methods had been established for studying renal function and we pursued these avenues. Chemicals for these analyses

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required a certain degree of purity and only relatively crude chemicals were available for some of these procedures. Therefore, we spent considerable time making the proper chemicals ourselves. In addition, certain intravenous solutions were required and many were not available from pharmaceutical and drug houses. Here again, we were obliged to purify chemicals and subject them to animal tests to ascertain their purity prior to conducting tests with human subjects.

Since only a relatively small number of subjects had been tested with these procedures at this time, the powers in control at the NIH suggested in the early forties that our efforts might be better spent on studies beneficial to the war effort.

It was about this time that the members of the vitamin B complex and associated vitamins first became available to the medical profession. Not too much was known about the merits of these new vitamins. So, we decided to enter into this field. That's when things really started jumping. The reason jumping is the appropriate word is that the initial test animal chosen was the green leopard frog (*Rana pipiens*).

The method used involved the frog being pithed, mounted on a vertical board, with the gastrocnemius muscle exposed at the lower extremity. A muscle lever was attached and the muscle stimulated with an electronic stimulator and the muscle level tracing recorded on a smoke kymograph drum. Control studies were carried out followed by a perfusion of the animal with the substance being tested. Approximately 10 animals were included in each test.

It was about the this time, in the summer of 1943, that I received greetings from the President with the information that I would be inducted into military service. In November of 1943, since a number of our experiments were still to be done, we were kept busy wrapping up the experiments before my departure, often working until midnight. Luckily, Dr. Shock was able to recruit an assistant to replace me until my return in November of 1946.

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When I came back I was pleasantly surprised to see that we had more commodious working quarters. The unit's personnel had increased. In addition to Dr. Shock, there was a secretary, an M.D., and two technicians.

The unit was now housed on the ground floor of a vacant ward in the chronic section of the City Hospitals. New laboratory furniture was procured and, with time, the laboratories and offices were ready to go.

Immediately upon my return, even more personnel were hired. Within the next year or so, investigators in various fields came onto the scene. Some of the disciplines represented at the time included carbohydrate metabolism, cardiovascular, endocrinology, nutrition and metabolism, psychology, and renal function.

It was also around this time that Dr. Shock published his first volume of *A Classified Bibliography of Gerontology and Geriatrics* (Shock, 1951), which can best be described as the key to the world's literature in the field of aging.

The National Heart Institute was established in 1948 and we were later transferred to it from the Experimental Biology and Medicine Institute (which had developed from the old Division of Physiology). The budget increased with the numbers of personnel and this was when NIH came into its own.

Science magazine recorded a typical interview by a Congressional committee with an institute director in the following manner:

"Now Doctor, you have presented to us your budget for X number of dollars in which you postulate that the following experiments can be conducted. Tell us Doctor, just what could you do if you had X more dollars?" Truly those were the "Golden Days of NIH."

In the early 1950s, it became evident that the human organism was only capable of contributing so much to the study of aging. Consequently, a section was set up in biochemistry and enzymology and our first animal colony was established.

The Unit again increased in size at a relatively slow rate with new sections being added in fields such as molecular biology, cellular metabolism and biochemistry, to supplement ongoing human studies.

In the latter part of the fifties, the Baltimore Longitudinal Study of Aging came into being. This study, of course, continues today and a recent book, *Normal Human Aging: The Baltimore Longitudinal Study of Aging* (Shock *et al.*, 1984), highlights some of its accomplishments over the first 23 years. With the addition of these new sections and the human aging study, we were bulging at the seams. Some of the newer units had to be housed in other buildings at City Hospitals. We occupied the entire ground floor of the abandoned ward. The second floor housed our research ward and laboratories concerned with human research.

It became evident that space was a limitation to further expansion. Then, Dr. Shock, ever the skillful diplomat, and others approached Congress with our needs for more space. The final result was the present Gerontology Research Center, affectionately known as GRC. This building was completed and dedicated in 1968 and this allowed for the further expansion required for aging studies.

In late 1965, the GRC became part of the National Institute of Child Health and Human Development. In 1972, I reached age 65 and retired from the Center. Reflecting back, it became quite obvious to me that we had facilities rivaling those of most of the NIH institutes in Bethesda.

The only logical outcome of all this was to create an aging institute. Thankfully, Dr. Shock was able to realize the final act of what must have been one of his fondest dreams when the National Institute on Aging was established in 1974. Of course, the first part of the dream had already come true with the completion of the GRC in Baltimore.

Dr. Shock continued to direct the Center and became the first scientific director of the new institute, serving until his retirement in December 1976 at the age of 70 years. He subsequently was appointed NIH Scientist Emeritus and still continues to take great interest in the institute's progress. He spends at least four days each week at the Center writing papers, monographs and consulting with the staff.

In looking back over the years, I realize how fortunate I was to have been associated with such a remarkable person and scientist as Dr. Shock. He is a man who has been extremely dedicated, very perservering, and persistent in seeking his goals. I think the present research building and the institute are living monuments to Dr. Shock's contributions and commitment to the entire field of aging research.

Acknowledgments– The authors gratefully acknowledge the editorial assistance and manuscript preparation of Eloise D. Acord.

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BITS OF FOSSILS

JOSEPH T. FREEMAN

1530 Locust Street, Philadelphia, Pennsylvania 19102

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A MEETING

HE WAS craggy, old, and in the Anglo-Saxon term, grizzled. His frame was big in a settled way with wide shoulders, deep chest, and heavy neck fixed on its base like an old building that has worked its way onto its foundation.

We exchanged courteous smiles as we started up the stairs together at the 1958 annual meeting of the Gerontological Society of America. At the bottom level, we were strangers, but our friendship had begun by the time we reached the top. It was in that quick friendship that this story of the Fossils, née the Cliffhangers, begins.

AN EXCHANGE OF VIEWS

As we walked up, he put out a hand and slowed his pace: "Getting older, you see; have to take it a little easier." After one flight, he stopped to get his breath. He needed the useful pauses of semicolons in his sentences. I noted with a glance that his ankles were swollen.

After a bit, he asked who I was and what I did.

"I'm an internist interested in geriatrics and gerontology as well as in this Society's activities."

"Good. I'm Dr. W.W. Peter, retired Public Health physician, and involved in some of these things myself," adding with a laugh, "obviously in a personal way, too. By the way, do you know Dr. Shock?"

"Very well, indeed," I replied. "He's a friend. I know of many of his activities in organizations, and particularly his studies of the aging process."

"Just so. Perhaps I can make some contributions to his work."

"In what way? His papers on the physiology of aging that are standards of precise reporting already have established many norms."

"True, and that is why I am working on a plan. Take my card; I live in Maryland. Write

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to me and I'll respond promptly. But I'm forgetting. Just what studies are you doing now?"

"Better to say, a study that I'm trying to get under way," I said. "It's a survey of sex in the elderly. I'm not far along with it as yet."

"In time. Perhaps I can be of some help to you, too. Tell me about it in your letter."

"I'll remember . . . Perhaps we can meet again before the session is over. Meanwhile, permit a liberty. Are you pushing yourself a bit?"

Turning to go, he smiled. "I guess I get carried away here" (touching his head), "and forget the protests from here" (touching his chest). "Since you mention it so tactfully, perhaps you would like to offer a quick judgment of my condition."

"That's too much of a challenge on such short notice. I wonder if any one would dare risk an answer? But you know how we physicians form opinions. It's called roundsmanship, and it's a dangerous game to play."

"Certainly. I do it myself. Listen, when you write to me, send me a list of diagnoses of what you think about my condition. I'll let you know how close you come to the mark. I've been under the care of an excellent internist who keeps me fully posted. Perhaps too fully," he concluded a little wryly.

"Well, it will have to be considered. One more minute. Can you tell me a little about what your proposals are for Dr. Shock?"

He took a new lease. "It's a long story, and I'll write to you about it, but the point is that I have been talking to a number of my colleagues, all retired professionals, to see how we can make a contribution of ourselves to the knowledge of the aging process. I've made it a point to inquire about Dr. Shock's interests, studies, and commitments, and I like everything that I have learned." He paused for a breath. "I got in touch with him with the hope that he would be receptive to my idea. Our group would be willing to undergo regular lifetime total body evaluations starting now. Serial studies of this fairly stable population would permit the formation of data on which to establish some principles of aging's mechanisms in men.

"At the start, we called ourselves the Cliffhangers. As you see the sample in front of you, namely, me, the term is right. Perhaps my present address, Scientists' Cliffs, put me on to the name. Later it seemed better to call ourselves the Fossils, which is more appropriate. That's it in a nutshell."

He interrupted himself to say: "By the way, you won't forget to send me that list of diagnoses?"

"That's somewhat intimidating," I replied. "It would be brash, and at best an educated guess, if that, but I'll do what I can. It would be far easier for us to forget about it."

He laughed. "Of course, but don't worry about it too much. Just send it along. You have my address. I'll be looking forward to your letter. Eventually I'll let you know how you did."

With that and a handshake we parted. We exchanged letters but never met again. He died suddenly four months later. Perhaps he had a degree of awareness, because he said that he would see that my clinical opinions would be compared with more seasoned observations. He had arranged for me to get a copy of his autopsy report. (Was that the "eventually"?) The results were fairly in agreement with my diagnoses, which would have pleased him. I have reported this strange incident accurately, because it is important to the knowledge of the man and the part that he played in a distinguished longitudinal study of aging.

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DR. WILLIAM WESLEY PETER

Dr. W.W. Peter was born in Elliston, Ohio, December 1, 1882. His medical degree was awarded by Rush Medical College of the University of Chicago in 1910. He married Elizabeth Whipple of Chicago in 1911. After studies at Harvard, MIT and Yale he became a Doctor of Public Health in 1918 and received a Master of Public Health degree from Harvard in 1947. His internship was served in Toledo, Ohio, after which he had a long public health career in China, including service as Medical Officer with the Chinese Labor Battalion in France 1917–1919. In 1934 he was appointed Medical Director of the Navajo Area in the United States Department of Interior's Office of Indian Affairs; he served there from 1942 to 1945. Later he was an associate professor of public health at Yale, and then as Professional Lecturer in the same field at George Washington University until 1949.

On retirement, he moved to Scientists' Cliffs, Port Republic, Maryland, where he died March 31, 1959 (Directory of Medical Specialists, 1957; Obituary, 1959).

LETTERS

Excerpts from letters and documents tell of Dr. Peter's vision and of his dedication to an important work on aging. He had the insight to bring it to the attention of Dr. Nathan Wetherill Shock who was able to elaborate the plan and to develop procedures for their realization.

I acknowledged our meeting at the session of the Gerontological Society of America in a letter sent to Dr. Peter November 11, 1958 (Freeman, 1958a). I expressed the wish that he would find a way to mobilize his Fossils along the lines that he planned in association with Dr. Shock.

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I took the opportunity to ask whether he could assist in the study on "the sexual capacities, habits, and constitutional make-up of people from sixty up. This has been a sadly neglected field," I said. "And I wonder if you and your fossils would be willing to answer some frank questions on an anonymous basis in order to arrive at some type of cross-section of opinion of this controversial field." I intended to distribute a questionnaire to 200 people that would be the basis of a report to be read at the Society's 1959 meeting at Detroit. The recipients of the form would be Dr. Peter's colleagues and other whom he had entitled the Cliffhangers or Cliff Dwellers.

Within three weeks, on December 1, 1958, he answered that he would try to further the project early in 1959. [The plan did not succeed, but the research was done in Philadelphia and was published as "Sexual Capacities in the Aging Male" in 1961 (Freeman, 1961), and as a chapter in several editions of Cowdry's *Care of the Geriatric Patient* (Freeman, 1968).

In the same letter again he requested the list of diagnoses that had been part of our conversation in November. His persistence on this score was unusual and challenging. Immediately I drafted an attempted at a reply.

> Philadelphia, December 1, 1958
>
> Your pin-pointed question about a long-distance diagnosis puts me on the spot. It reminds me of the old stories about Doctor Libman of New York, who was reputed to have taken students into a subway and to have pointed out diagnostic features of other

denizens in the car. There is very little danger or responsibility in this type of sophistry. Other fare-paying individuals did not have to affirm or deny the accuracy of the good doctor's observations. However, I must respond to your challenge for right or for wrong.

My letter enumerated seven conditions about Dr. Peter's health as I assumed it to be at our meeting. The list was given in systematic order and the letter concluded with a final paragraph in which I again expressed concern: "I was almost going to add the possibility of an esophageal hiatal hernia, but my area of error above cited is so enormous at this point, that I dare not go beyond the realm of reason. As a matter of fact, most of this is guesswork, and not reason, so I know that you will make allowances for superficial observations" (Freeman, 1958b).

His reply was immediate.

December 6, 1958

By your letter of December 1st you have lived up to your end of the bargain with unexpected perspicacity as will be apparent when you check your impressions with the findings made more deliberately and with the help of accepted diagnostic techniques. Now I live up to my offer and send you herewith copy of the November 14, 1957, Narrative Summary given me by Dr. Daniel J. Daley, USPHS Medical Director, and Medical Officer in Charge of the USPHS Hospital, Baltimore. He lists only five diagnoses. You list seven and were tempted to add yet more but restrained yourself. Some of the items you included may be verified only at post mortem. For this final study all arrangements have already been completed, thanks to the help given me by Dr. Nathan W. Shock. Both our family physician, Dr. Page C. Jett, and our family mortician have been instructed in detail to hasten the transportation of my body to the Baltimore City Hospitals Pathology Building after I have finished using it. Dr. Shock or pathologist Dr. Abou D. Pollock should be asked for a copy of the PM findings if you are interested.

As soon as you have prepared your questionnaire, send me several copies for discussion with my inner circle of male friends here at the Cliffs on how far we can go in helping you in acquiring 200 respondents.

(signed) W.W. Peter
(Peter, 1958a)

STAGES OF THE DEVELOPMENT OF DR. PETER'S IDEAS

Significant parts of a letter from Dr. Peter tells of the progress of his ideas for a program of longitudinal studies of an aging cohort. From his home, he wrote of various plans that he had entertained and of his ways to bring them to resolution.

January 10, 1959

In reviewing my files to begin the New Year, I came across an interesting newspaper notice which I followed up in two ways. I enclose a copy of the newspaper clipping announcing a study by the Department of Health, Education, and Welfare, of retired people. So on November 27, 1957 and before Dr. Nathan W. Shock accepted me into his fold, I wrote Secretary Marion B. Folsom, offering to be one of the 3000 he would "study" for as long as I lasted on this mundane sphere. . . .

The Assistant Director of the Bureau of Old Age and Survivors' Insurance had to decline Dr. Peter's offer because "they were accepting only retired people receiving Federal old age and survivors benefits . . . I was either too fortunate—or to unfortunate to be eligible."

He took another approach as the result of learning that Mr. Charles S. Lavin was the Executive Director of 15 retirement hotels in Florida, California and elsewhere. "I estimated he must have hundreds of retired guests none of whom might be eligible to participate in the Folsom study" (Peter, 1959).

In response to a letter November 30, 1957 to Mr. Lavin, whose work received national publicity as the result of a magazine article, Dr. Peter received a letter on December 6, 1957 stating that the notice of the projected research plan had been posted in each of the facilities. "That is the last I heard about him or about the Folsom study" (Peter, 1959).

Dr. Peter described his plan to his colleagues with an invitation for them to be participants in the longitudinal study:

Scientists' Cliffs
Port Republic, Maryland

Dear Neighbor:

May I suggest that you pause and read the enclosed description of a research project in which I hope you will consider it worth your time to participate. Several of us are discovering that having a part in such an important scientific program is both interesting and rewarding.

There is a reason why people like us should volunteer. We are not receiving custodial care and we are not living institutionalized lives. Nor are we medically indigent. Such research material, if too preponderant, distorts the picture. Many of those being cared for in the Baltimore City Hospitals and the Old Peoples' Home (600 residents) have been buffeted by the storms of life for so many penilizing years that they present more than the average number of abnormal findings; both physical and psychological. More persons are, therefore, needed from the outside who live normal lives in their respective communities. During my two sessions in Baltimore, Dr. Shock and his staff often mentioned their concern over the present imbalance of research material. When I toid him I lived in a community of educated, active, self-sustaining, social minded people, Dr. Shock reacted as I hoped he would. He opened his door—wide! Hence, this invitation.

What would your participation involve?

1. Securing your physician's approval to take the entrance examination and the series of aging process tests if you are accepted.

2. Spending one day at the Baltimore City Hospitals to take the required physical-medical examination, given by physicians of the Gerontology Branch (10:00 a.m. to 3:00 p.m., with lunch provided in the Hospital). Your having known ailments need not deter you from volunteering. I have several, yet I was accepted. The examination is thorough and includes electrocardiogram, chest x-ray, blood and urine analysis, and other procedures. It is especially worthwhile for anyone who has not had one recently. Occasionally, unsuspected pathological conditions are revealed in the beginning stages. All abnormal findings are reported to your physician, since the Gerontology Branch staff will not treat any diseases found. They accept you as you are, or reject you.

3. After returning home to await the report of your entrance examination and, if accepted being willing to return to Baltimore to take the series of aging process tests at some time mutually convenient. This schedule lasts from 10:00 a.m. on Day I, all through

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Day II, and until 3:00 p.m. on Day III, with no free time for outside shopping, visiting, or evening theater. You will occupy a guest room for two nights. At present, only men can be accommodated. Later it is hoped to have guest rooms for women participants. Prescribed food and liquid intake will be provided.

4. Agreement in principle to repeat the testing procedures annually for purposes of comparison, insofar as this can be arranged.

5. Provide your own transportation to and from Baltimore. Some prefer to go by twos.

There are no charges for the above items 2, 3, or 4. To save your time and telephone tolls to Baltimore, I have agreed to share my own experiences with any who may be interested, answer questions, and make the appointment for your physical-medical examination. My telephone number is Prince Frederick 33 W 1.

Sincerely yours,
W.W. Peter, M.D.
(Peter, undated)

Enclosure

Subsequently the proposal was supplemented by another letter to the men whom he wanted to become part of the project directed by Dr. Shock.

December, 1958

Dear Friend of Gerontology:

1. From my 80′ high, glassed-in porch overlooking the western shore of the Chesapeake Bay, I am writing you the first of what I hope may be a series of newsletters about the gerontological research program of which you and I and about 100 other men have become a vital part. From a very modest beginning in February, 1958, our group of volunteer participants has increased to such size that some method should be employed to keep us informed, interested, and of a mind to help wherever possible. It was felt that there might be something of even greater importance than the personal benefit each of us received from a high quality physical/medical examination followed by whatever contribution we made when later we took numerous aging process tests (most of which it was not necessary for us to fathom and understand!). Nor that our full contribution to the program would end when in subsequent years we responded to being called back to the laboratories for repeated tests for comparison and for whatever new tests might be prescribed by the scientists with whom we were trying to cooperate. But the most important reason of all for instituting occasional communications between the gerontological research center and our group of volunteers is the fact that Dr. Shock is so gratified over the experience of this first year that he hopes to continue the new policy of depending upon actual participants to bring in new ones. It is hoped that occasional newsletters, written by one member of the group for the other members, may help develop a group spirit in support of this particular research program which is of such difficulty and importance to future generations of mankind.

2. With some of these thoughts in mind, I volunteered to try and represent our group. Please do not think this is an act of presumption on my part. No means exist within our group to effect an election. We are not organized. I just up and volunteered and was graciously accepted by Dr. Shock. In my favor was the fact that there were no other candidates at the time! I may have some misgivings, not entirely related to modesty, but I shall do the best I can.

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3. In my favor also is Dr. Shock's offer to try and help me in every way he and his staff can by providing pertinent material from time to time, and by being critical all of the time. Each of you is hereby invited to take the same attitude for a common good. Together we may make something worthwhile out of this thing. Let's give it a try!

4. The first subject I want to discuss is the recruiting of future subjects. On October 29, 1958, Dr. Shock wrote me as follows: "I think your idea of a Reserve Corps of Volunteer Subjects is excellent. If we had a reservoir of acceptances, we could get in touch with them a month or so before an opening appears on our schedule and plug any holes that may develop because of conflicts in schedules on the part of the subject."

5. There were in all 76 volunteer subjects (aged 23 to 97) from the outside who were scheduled at the rate of two per week for calendar year 1958. New ones are already scheduled well into 1959. There is no immediate urgency for new recruits. But to assure Dr. Shock and his staff that an adequate and steady influx of new subjects will be forthcoming is something in which each individual in our present group can participate in achieving.

6. To help any of you who aspire to the satisfaction of "educating" men of your acquaintance to consider "joining up," drop me a line and I shall be glad to send you a copy of the Peter/Shock outline describing this splendid project, or send me your friends' names and addresses if you do not have time to do the preliminary spade work in recruiting them. Those who volunteer will be added to the Reserve Corps of Volunteer Subjects for Gerontological Research.

7. Join me in being proud of the fact that at the 11th Annual Meeting of the Gerontological Society, Inc., Dr. Nathan W. Shock was unanimously voted in as President-Elect. These gerontologists don't believe in rushing a man into office precipitously. He assumes the Presidency in 1960!

8. Will you kindly participate in the "Suggestions to the Editor" Department? For one thing, would you like to know the composition of our present group? If so, will giving name, address, profession, and present age suffice? Our group includes many whose sketches may be found in *Who's Who in America*, *American Men of Science*, and other volumes of biographies. So if we should send you a list, guard against its falling into the hands of hucksters who would just revel in the chance to circularize us about their wares.

Fraternally yours,
W.W. Peter, M.D., Dr. P.H.
Scientists' Cliffs
Port Republic, Maryland
(Peter, 1958b)

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Dr. Shock explained the project as follows to the volunteers who had responded to Dr. Peter's letters:

THE GERONTOLOGY BRANCH
of the
NATIONAL HEART INSTITUTE, National Institutes of Health, U.S. Public Health Service, Department of Health, Education and Welfare
and
THE BALTIMORE CITY HOSPITALS

1. The Gerontology Branch is a joint operation, as indicated above. The 15 investigators currently on our staff include physicians, biochemists, zoologists, embryologists, histolo-

gists, physiologists, and psychologists. These investigators, along with 25 technicians, are permanent appointees to the staff of the National Heart Institute. The Baltimore City Hospitals provide laboratory space, nursing services, and all necessary operating utilities for the program. We are engaged in research on the problems of aging. Why? According to the Bureau of the Census, our population in 1900 was 75,994,575. Included were 3,080,498 people aged 65 or over, or 4.1% of the total. By 1950, our population had doubled approximately to 150,697,361 but the number of people aged 65 or over had quadrupled to 12,271,178 or 8.1% of the total. (By 1975, the Bureau estimates that the percentage will be 9.4%. A parallel fact is that the average age at death has been increasing steadily in the USA from 35 years in 1879 to 67.5 years in 1955 for white males, and 73.7 for white females). This rapid increase in the number of elderly people in our population makes it imperative that we learn more about the effects of increasing age on the biochemical, physiological, and psychological characteristics of older people.

2. We need to know a great deal more about the performance capacities of adults of all ages. Furthermore, we need to know more about the basic biological processes of aging in order to make recommendations that will minimize the disabilities of old age. Thus, the ultimate goal of the research program on gerontology is to discover the factors that will reduce the incidence and severity of the disabilities that now plague elderly people. The goal of research on aging is not simply to prolong life, but to make it more effective and meaningful.

3. The Gerontology Branch carries out studies on the basic biology of aging. These studies are concerned with cellular physiology and may utilize simpler animal forms such as bacteria, flies, mice, and rats, since aging is a property that is common to all living things. These forms of life are useful because experimental controls can be maintained more easily than in complex subjects such as humans. Much of our knowledge about how living cells work and age will come from these studies.

4. In addition to these studies on cellular processes, we must also learn something about the performance capacities of the total person. These studies on age changes in the human constitute an important part of our program. We are evaluating the functional capacities of various organ systems. Examples of the tests are estimates of lung function, heart function, kidney function, basal metabolism, speed of reactions and responses to standardized exercise. In addition, studies on psychological and psychomatic functions are in progress.

5. Up to now, we have tested individuals of different ages (20–90 years) and can characterize average changes with age. The older subjects for these studies have been selected from individuals receiving custodial care in the Old Peoples' Home of the Baltimore City Hospitals. Although we have obtained important baseline data on these subjects, we need to expand our studies to include observations on individuals who are still living in the community. In addition, we need serial observations on the same individual if we are to understand fully the aging process.

6. To this end, the Gerontology Branch is requesting the cooperation of individuals in the community to serve as subjects for study. Volunteers for this program will be performing an inestimable service for the science of gerontology immediately and ultimately for their fellow men.

7. It is anticipated that tests will be repeated on an annual basis. In addition, each subject will be offered the opportunity to participate in other research programs of the Gerontology Branch.

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8. It is recognized that these studies will require many years for their final completion. However, the important social problems of an aging population make it imperative that we start now to obtain essential information. The staff of the Gerontology Branch is dedicated to the solution of these problems but their success depends upon widespread public understanding and participation.

Nathan W. Shock, Ph.D.
Chief, Gerontology Branch
(Shock, undated)

SALUTATION TO A SCIENTIST

Doctor Peter did not live to become a long-term part of the National Institute of Health Study.

Norris and Shock (1960) in what then was the Gerontology Branch of the National Heart Institute, wrote an appreciation of Dr. Peter's contribution to the longitudinal studies of aging. Dr. Peter's views of October 1957 were quoted: "There is a reason why people like me should volunteer. We are not receiving custodial care and we are not living institutional lives. We are not medically indigent." Studies of people "who live normal lives in their respective communities" offer contrasts "to studies done on people of like age who live in various health and supervised facilities in which there are implications of medical conditions."

His proposals were received favorably. By 1958 a group of his colleagues and their friends had been enlisted as volunteers for sequential evaluations. As Norris and Shock said, this "articulate protagonist for gerontology" properly received "credit for putting order into the dream of gerontologists" (Norris and Shock, 1960).

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The epilogue of this narrative was the 1984 publication by Shock and his associates, *Normal Human Aging: The Baltimore Longitudinal Study of Aging*. The volume that summarized the work of the Baltimore Research Center of the National Institute on Aging (successor to the earlier Branch) was dedicated to Dr. Peter. His picture graced the front of the book (Shock *et al.*, 1984).

The authors said that this summary of a quarter century of work by a number of investigators was a reflection of Dr. Peter, "whose foresight and enthusiasm played an important role in the recruitment of subjects . . . He had seen the need to study aging in individuals who were free of disability diseases and were leading successful lives in the community." The authors, whose careers of research were projections of his ideas, stated that the volume "stands as a memoriam to his vision."

Dr. Nathan W. Shock has had long service in gerontology in the National Institutes of Health. His scientific training and talents were augmented by his ability to meet step by step the practical issues that were necessary for the creation of an organizational structure in which to undertake longitudinal studies of aging. One physician's far-seeing concepts of 1957 were put into motion by a perceptive research scientist.

By 1984, an understanding of the many dimensions of human aging were expanded in studies that have achieved continuity.

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THE BIOMEDICAL CHALLENGE OF THE NEXT HALF CENTURY

The Search For New Knowledge of the Aging Process and its Associated Disease

IRVING S. WRIGHT

President, The American Federation for Aging Research, 335 Madison Avenue, New York, NY 10017

Preface

It is a privilege to contribute to this volume of Experimental Gerontology published in honor of Nathan Shock. He has served as a leader and inspiration to all of us who have been involved in the development of the field of geriatrics/gerontology for nearly a half century. He has trained many of the most distinguished of the present generation of teachers and scholars in this field. The country owes Nathan a great debt for his outstanding contributions to the present and future health of its senior citizens.

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TO HAVE lived as a physician, investigator and teacher of medicine through the past 60 years has been an extraordinary experience. No other period in world history has witnessed such biomedical advances. In the 1930's, the great killers and cripplers were the infectious diseases predominant in the United States, now in large measure controlled or eliminated. Our wards were full of patients dying of pneumococus pneumonia, meningitis, typhoid fever, rheumatic heart disease, tuberculosis and polio. These were rampant as were measles, scarlet fever and mumps, all potentially serious.

With the great step forward, made possible by the antibiotic and vaccine era, the population aged a decade or so and the new predominant diseases became athersclerotic heart disease, the wide variety of cancers, hypertension with renal disease and strokes.

As in the infectious disease era, physicians were restricted by lack of knowledge to limited preventive and therapeutic approaches—largely palliative. Once again, as in the case of infectious disease, it was found that the care of the patients using *available* knowledge was inadequate and that it was absolutely essential to develop an army of research oriented scientists concentrating on these specific problems. This was accomplished and supported

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by the National Institutes of Health, the American Heart Association, the American Cancer Society and many of the voluntary centers and agencies.

After 40 years it is possible to regard the results with considerable satisfaction. Research has step-by-step revealed new findings that when applied have reduced the death rates from heart disease and hypertension with stroke and also that of a variety of cancers. The task is far from completed and research in these areas must continue to be supported while the new knowledge must be applied. Application is often as difficult to achieve as the original work; witness the problems of tobacco and obesity. It became clear that with the decrease in the incidence and effects of the infectious diseases, heart disease and some forms of cancer, the aging process itself required more intensive study.

Among the few who recognized this need was Nathan Shock, who played a leadership role in the development of the Baltimore Longitudinal Study. He recognized the advantage of following individuals throughout their life span to observe and record the changes associated with aging. To Nathan Shock, his co-workers and students, many of whom are now distinguished teachers and scholars, we owe one of the worlds greatest data banks on the many faceted phenomena associated with aging. New findings continue to come from this great study.

As a result of continued prolongation of the average life span to 70–78 years, a new spectrum of health problems has arisen. Diseases which have existed for centuries but did not present great demographic health problems when the average age expectancy was 40–50 years have now developed into the major health challenges of today and predictably far into the 21st century. We are faced with diseases which are disabling millions of our citizens, too often for years before the terminal event. If we consider the diseases that disable a million or more of our elderly and for which we lack the knowledge necessary to prevent, cure or even treat satisfactorily, the list is long and formidable. To mention a few: osteoarthritis, dementias including Alzheimer's Disease and other diseases involving the nervous system, loss of immunity against infectious diseases and cancer, blindness due to macular and retinal degeneration, progressive renal disease, incontinence and many others. Once again we are faced with the fact that critical knowledge is lacking.

We must now develop a large cadre of scientists and physicians who will concentrate on basic and solid clinical studies designed to secure and apply the knowledge we need. Social and economic studies and new buildings for care, valuable as they are, will not supply this key information. Biomedical research is the *only source*. Greatly increased support must be made available now and in the immediate future. Otherwise little progress will be made and a decade from now our prevention and treatment of these diseases will still be unsatisfactory while the magnitude of the problem will multiply at an overwhelming rate.

From its founding in 1980, the American Federation for Aging Research (AFAR) has focused its objectives on research in all biomedical aspects of aging and the associated diseases. Findings from a study using one discipline or involving one disease may be of importance to others—but often the cross-communication is retarded by the common custom for reports of new findings to be presented within specialist groups or societies.

Applications for grants from AFAR have been accepted from researchers working in all related disciplines and from more than 100 centers throughout this country. Of the first 70 awardees, 38 have been under the age of 40. It has been of interest to review the subjects under investigation by the first 70 AFAR Awardees. They may suggest areas which appear to be challenging to new researchers.

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Since the grantees are working in 46 different institutions, they represent a broad spectrum of the interests of the new generation of researchers in this field.

Of 154 applications received in 1985, 25 were awarded grants. Another 25 excellent projects would have been supported if funds were available. While heart disease and cancer are leading disabling and fatal diseases of the elderly, they are well funded and AFAR has emphasized less well supported areas requiring intensive study.

As has been noted in other biomedical research centers, the grant applications of AFAR indicate an increasing proportion of Ph.D.'s and fewer physicians. The reasons for this are of interest and probably multiple.

1. More young Ph.D.s are becoming involved in biomedical research.
2. The basic problems related to aging can be studied productively by Ph.D.s. They are often better trained in such disciplines as molecular biology and genetics.
3. Physicians interested in aging are in great demand to assume responsibility for the development of geriatric programs dealing with aging. This includes organizing and operating clinical facilities and teaching responsibilities in medical schools. There are far too few well trained candidates to meet the need. Therefore, these assignments are being made increasingly attractive.

At the same time, it is regrettable if physicians are indicating lessened interest in the basic sciences since in the ultimate, the application of new findings is most often their responsibility. Both groups should have training and experience in research in order to properly coordinate the application of new knowledge of this type. It has also been my observation that clinicians who have had research experience are better able to evaluate the findings reported in the Scientific and Medical journals.

Some areas now have special appeal–for example, genetic studies and modifications, molecular biology which is inexhaustible as it reveals the variations in each type of tissue

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and the modifications with aging and disease. Yet key findings may change the future lives of a major population group.

There has been increasing interest in genetic medicine and the early recognition of specific heritable conditions and the identification of familial risk factors for later disorders. New techniques including chorionic sampling, fetal visualization, gene transfer and cloning methods are being pursued. Many of these studies concentrate on the abnormal physical and mental states that are detectable early in life.

There are genetic traits which persist into old age such as the many aspects of physical appearance. The potential chronological or time span of our existence is of unique interest but little understood. The fact that some families are known to be short lived and others for their longevity suggests that we are born (or conceived) with a longevity which cannot be exceeded by any known means today. Because of the variables in life—disease, malnutrition, drugs, violence, war, and natural diseases—one must consider these factors before drawing conclusions. Nevertheless, 60 years of observing families suggests strongly that individuals do, under so-called normal conditions, progress to aging under quite different biological timing. How early can this be evaluated? With the exception of Progeria and a few other rare examples we lack the information we need for this prediction. It now seems likely that while we cannot prolong human life beyond its maximum genetic potential (there are those who believe this may be possible in the future) our life span is shortened by the variables above mentioned. Will markers be found to indicate the biological potential? Recombinant DNA and allied technology has led to remarkable diagnostic achievements with muscular dystrophy, cystic fibrosis and a few other disorders but whether this type of approach will yield the information we need to foretell the rate of aging in individuals is questionable and certainly not in the immediate future. This is a challenging subject for study.

Nature provides us with an extraordinary experiment for which we do not have an appropriate experimental model, Progeria, with aging at close to 10 times the normal rate and the diseases including myocardial infarction, strokes and other physical changes associated with aging. This is related to biological acceleration, like a speeding clock but where is this remarkable control system? What intracellular mechanisms initiate and sustain it? If it is in the brain, what area or nucleus? How can it be regulated? Critical information regarding this remarkable phenomenon, if available, might be applicable to the rate of aging of a much larger proportion of our population as noted in individuals or families who die young versus those with remarkable longevity.

Dr. W. Ted Brown is carrying on extensive biomedical studies working with Progeria patients and their families in the development of an International Registry, initiated by the American Federation for Aging Research and financed by the Bedminster Fund. This remarkable phenomenon justifies much more intensive study.

The National Institute on Aging is still young and therefore has not received financial support comparable to the older national institutes, notably the Cancer and the Heart, Lung and Blood Institutes even though the problems it is expected to resolve will strain our financial and health resources into the foreseeable future. Funds for training, research, teaching, care, scientific and medical personnel are sharply limited. An appropriate adjustment in national focus concentrating on research and training in this field is absolutely essential to our future.

In the private sector, it is encouraging to see increasing interest in aging research by foundations and corporations. Large foundations, including the new Hughes Medical In-

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stitute, have the opportunity to make major contributions, especially as they concentrate in special fields. It now appears that most of their support will be in large grants to major institutions. However, some of the great discoveries have been made by less heavily endowed investigators. A modest grant can often enable a young researcher to embark on a highly productive career. Their original observations, or the workers themselves frequently later migrate to more sophisticated centers.

What proportion of effort should be allocated to basic studies of natural phenomena and how much should be concentrated on targeted research? As budgets tighten, the pressure increases to concentrate on targeted research aimed at a quick and specific human benefit. Yet we are reminded that outstanding discoveries have resulted from free flowing research, often from serendipity. Therefore, both types of research should be encouraged.

CONCLUSIONS

There are certin critical areas that demand particular attention. They include:

1. *Genetic Studies*—General studies of genetic backgrounds and traits are of great importance. But genetic engineering must be approached with the greatest of caution. The long term results may be highly beneficial, but we are entering new territory and the results may also be dangerous, even catastrophic—very careful control is essential.
2. *Neuroscience*—The need for new knowledge here is pressing with the increasing incidence of dementias, including Alzheimer's disease, Parkinson's and also the many less known central and peripheral neurological changes associated with dysfunction. These studies must include muscular and sensory changes, loss of vision (macular and retinal degeneration) and deafness, each involving millions of citizens.
3. *Muscular-Skeletal Diseases*—Notably osteoarthritis, osteoporosis and Paget's disease.
4. *Immunological Loss*—with aging against both infection and cancer.
5. *Metabolic Regulation*—including both temperature, utilization of the wide variety of foods which we ingest. The lean muscle and fat distribution change with aging and hormonal activity.

All of these and other key phenomena must be studied at every level from submolecular activity, through molecular, cellular and finally to functional status.

Can we afford such comprehensive studies? The answer is that we can no longer afford to neglect them. Significant discoveries in any one of these areas can affect the lives of a million or more patients, helping to maintain them in reasonable health and leading meaningful lives until close to the terminal event. The stakes are very high.

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BIOGERONTOLOGICAL PRECEPTS OF NATHAN SHOCK WHICH INFLUENCED OUR AGING RESEARCH

CALVIN A. LANG AND JOHN P. RICHIE, JR.

Department of Biochemistry, University of Louisville School of Medicine, Louisville, KY 40292

Key Words: Nathan Shock, aging, Baltimore Longitudinal Study, gerontology

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INTRODUCTION

DR. NATHAN SHOCK has been a teacher to many and an inspiration from the time of our research collaboration 33 years ago to scientific counselling at recent annual GSA meetings. We are proud to represent two generations of Shock's students.

The best tribute and expression of gratitude to him is for us to present some of his key scientific principles that have been especially meaningful to our gerontological research. These precepts are personal selections; no doubt the list can be expanded considerably by others.

THE CLASSIFICATION OF GERONTOLOGICAL RESEARCH

Nathan Shock has been called a Father of Gerontology because of his pioneering efforts and singular devotion to carry out and promote gerontological research. To this end he has taught, developed, and encouraged a wide variety of investigators to elucidate the aging process from different perspectives. Particularly he has emphasized the essentiality of controlled, systematic, comprehensive, and uniquely multidisciplinary approaches.

A major contribution was his classification of the field of gerontology (Shock, 1951). This organization of diverse disciplines has had a widespread and continuing influence on aging research in its many forms. This organizational format was implemented by his leadership of the Gerontological Society (now the Gerontological Society of America) and the International Association of Gerontology. His insistence that these organizations should involve all aspects of gerontology was critical.

The classification of gerontology into four major divisions has served as a model in the design and development of many aging centers. The combination of biological sciences, clinical medicine, behavioral and social sciences, and social work seemed strange. However, it was a logical arrangement of biomedical and psychosocial categories that were subdivided

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into pure (biological and behavior/social sciences) and applied clinical research (clinical medicine and social work). In this way, all aspects of gerontology from science to practice were covered.

Several important sequelae were the result of this organizational format. First, the society meetings brought together scientists and practitioners from different disciplines and viewpoints. Thus, biologists in gerontology whose major experience was of quantitative, that is, objective measurements, became aware of the equally important, but difficult subjective data of psychosocial gerontologists. Reciprocally, this latter group became more aware of the importance of quantitative measurements in their fields. In addition, gerontology policy makers participated in these meetings that provided interactive forums in which to share results and needs. This indeed has been a splendid example of how to put science to work by crossing academic and political sectors. What other scientific organizations have provided this valuable opportunity to cross-fertilize?

Another important by-product of Dr. Shock's multidisciplinary format is the *Journal of Gerontology*, as an important and continuing endeavor. His devotion and enormous efforts to assemble a bibliography list for each issue (Shock, 1951) was a way to keep us current in an organized efficient fashion that helped to avoid the "rediscovery of the wheel" in our experimentation. His lists pioneered and antedated our now common computerized literature searches. His goal was the integrity of this important scientific journal.

THE BALTIMORE LONGITUDINAL STUDY OF AGING

Through his scientific and systematic approaches to gerontology, Shock provided us with several important research principles and findings which laid the foundation for aging research today.

Many of these contributions were a result of Shock's heroic experiment, the Baltimore Longitudinal Study of Aging (BLSA) (Shock *et al.*, 1984). Started in 1958, this study was designed to determine the effects of aging on physiological, psychological and psychosocial parameters in humans. It started with a number of men who were volunteers between the ages of 17 and 96 years, and has continued and grown through the present time to include women in 1978.

Until that time, a large data base of aging information had accumulated using a cross-sectional design. In this type of study, measurements are made once on a population of subjects covering a wide range of ages, so that only average differences between age groups are identified. There are several shortcomings of this experimental design such as the unavoidable natural selection of only "surviving" subjects in the older age groups, the so-called "cohort effects", caused by factors other than age which differ between age groups, and the increased incidence of many diseases found at older ages.

Shock recognized these shortcomings and selected a longitudinal design for the BLSA. In this study, sequential measurements of each older individual were made repeatedly at yearly intervals to identify aging changes in a self-controlled and more sensitive manner. Cross-sectional data on average differences among age groups also were obtained. Although this design obviates the problems of cross-sectional studies, it posed several practical problems such as high costs, subject recruitment and attrition, uniformity and quality control of analytical methods, data handling, and staffing. However, Shock identified these problems and through his persistence overcame them in this unique and ongoing study.

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To us his most important contribution was the concept of normative aging (Shock *et al.*, 1984). Shock realized the importance of separating disease effects from aging studies in order to identify the "actual" or normative aging process (Rowe *et al.*, 1976). This effort was difficult because of the incidence of diseases in many older subjects. However, by separately analyzing data from healthy, elderly subjects who served as controls, the mechanisms by which old organisms are at increased risk to disease could be determined. This concept also uncovered the need for new definitions of aging based on biological criteria rather than on chronological age based on time since birth.

Shock's findings established another precept of functional or biological age (Shock, 1972; Shock, 1980). These age scales are based on specific physiological and biochemical functions, such as renal clearance and cardiac output. Thus, a functionally or biologically old organism exhibits a decrease in functional capacity that is distinct from its chronological age. Our personal preference is the term, functional age, which includes molecular as well as physiological function levels.

Indeed, Shock also found that only certain biologic functions decrease during aging, while many others are unchanged. This differential effect emphasized the important precept that aging is not characterized by a homogeneous physical decrement. In fact only a few functions have been shown to decrease significantly in aging, and these few impairments may be responsible for functional age. One of the goals of the BLSA was to identify these critical biological functions.

Another important finding of the BLSA is that the biomedical and psychosocial characteristics of health elderly are the same as those in healthy young adults (Shock *et al.*, 1984). This definitive study corrected many of the misleading interpretations from cross-sectional results which indicated substantial and widespread declines in both biomedical and psychosocial parameters. Thus, the importance of focussing on normative aging without confusing disease effects was demonstrated clearly.

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However, despite the large amount of information from the BLSA and other longitudinal studies, all of the key aging indices have not as yet been identified. This important, but difficult, goal requires continued systematic investigation.

OUR AGING RESEARCH

Nathan Shock's teachings and experimental findings have provided a foundation for our own biochemical aging research over the past 25 years. Thus, in our early studies, where we were probing for biochemical changes that might be important in aging, it was necessary to develop and characterize our aging models. We used what we called a "3-M" system, which consisted of mosquito, mouse and man. To the best of our ability each model was characterized according to its life span characteristics and biological and biochemical makeup, and each model had unique biological and technical advantages (Lang *et al.*, 1965; Lang, 1967, Lang *et al.*, 1972; Lang *et al.*, 1973; Du *et al.*, 1977). Sampling was carried out in a systematic fashion at several ages throughout the life span periods with emphasis on maturity and senescence. This was essential to ensure that any changes found were aging-specific, and thus correlated with the period prior to or during the ages of increasing mortality.

As a result of this approach, our various findings and those of others led us eventually to the notion that the reducing capacity of aging tissues is decreased (Stephan *et al.*, 1966; Lang and Stephan, 1967; Lang and Acree, 1967; Du *et al.*, 1977; Mills and Lang, 1976).

Further, we found that this decrease is due to decreased levels of glutathione (GSH), the major intracellular antioxidant (Abraham *et al.*, 1978; Hazelton and Lang, 1980; Naryshkin *et al.*, 1981; Schneider *et al.*, 1982; Hazelton and Lang, 1984).

Our recent studies have focussed on this GSH deficiency of aging which we believe is of key importance because of the well-established and major role GSH plays in cellular metabolism and protection. Lower GSH levels occurred in every aging organism and tissue examined including our 3-M system, suggesting it is a general phenomenon. Its importance was confirmed by our finding that feeding low levels of GSH precursor caused an increase in both GSH levels and longevity in the mosquito (Richie *et al.*, 1983). Further this correction indicates the cause of the GSH deficiency is due to a lack of the amino acid, cysteine, and not due to deficits in enzyme or cofactor concentrations (Richie and Lang, 1985).

These results led us to the hypothesis that a GSH deficiency is a biochemical cause of the aging process. Our next step was to test this hypothesis in human subjects. In preliminary studies on male and female elderly of generally healthy, but undefined status, we found two distinct groups who were >60 years old, one with a high GSH level, the same as young 20–40 yr adults, and the other with low GSH levels (Naryshkin *et al.*, 1981; Schneider *et al.*, 1982). We believed these represent "biochemically young" and "biochemically old" sub-populations. These findings indicate that GSH may serve as a biochemical index of functional age.

To test this we initiated the Louisville Longitudinal Longevity Program (LLLP). This study was designed to determine quantitatively the aging changes of key physical, psychological and social health characteristics in a selected group of 52 women between the ages of 60–101 years. Most importantly these changes would be correlated with blood glutathione status.

The results of the BLSA guided us in the design of our study, which incorporated a multidisciplinary and longitudinal approach using validated analytical methods and documentation of subject ages. Also to eliminate the interfering effects of disease the subjects were preselected to be clinically healthy. These critical design features were essential to obtain meaningful results.

Although only in the fourth year, the LLLP results have supported our GSH hypothesis of aging. In the healthy elderly women, all physical health parameters determined were normal since they were in the range of Mayo Clinic, 1984 values for young and middle aged (21–55 years) healthy adults (Lang *et al.*, 1983; Richie *et al.*, 1984). Further, the psychosocial parameters including life satisfaction, well-being, and lack of depression, were substantially higher in this LLLP group than in an age-matched representative group from the Louisville area (Crews *et al.*, 1985). Most importantly, the blood GSH levels of all these women were high, and the same as found in healthy young adults (Lang *et al.*, 1983). Thus this differed from the high and low GSH subpopulations mentioned earlier. Collectively these results support Shock's contention that healthy persons are healthy regardless of age. Also the data provide further evidence for our notion that GSH is a biochemical index of functional age.

We believe the most interesting results from our study will come in future waves, as our group of LLLP subjects begin to show the "symptoms" of aging, which we anticipate will be reflected by a decrease in blood GSH levels.

Our research would not have been possible without the groundwork laid by Nathan Shock. His precepts guided us through our early days of groping to determine a fruitful and meaningful approach from the myriad of questions that needed investigation. Since then we have utilized the design and results of his BLSA to test our specific GSH hypothesis in

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human subjects. We still re-read with enthusiasm some of his papers from 30 years ago as these results have taken on new meaning in light of what is known today. This re-digestion of earlier work is essential for the complete understanding of current research results. For his guidance, findings and example we are indebted and grateful. May his labor continue to be fruitful through many more birthdays.

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INFLUENCE OF AGE ON CELLULAR DIFFERENTIATION: A T CELL MODEL

TAKASHI MAKINODAN, MEI-PING CHANG and NOBUHISA KINOHARA

Geriatric Research, Education and Clinical Center (GRECC), Veterans Administration Medical Center West Los Angeles, Los Angeles, California, and UCLA Department of Medicine, Los Angeles, California

Key Words: T cell, cellular differentiation, aging, interleukins, thymus, genetic alteration, bone marrow

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INTRODUCTION

IN 1972, NATHAN SHOCK recruited one of us (T.M.) to head the Cellular and Comparative Physiology Branch of the Gerontology Research Center at the National Institute of Aging (NIH). A high priority was placed on developing a program to assess the vulnerability of the cellular differentiation process of aging because at that time very little was known about this fundamental problem. Various cellular models of aging were developed, included the T cell model that subsequently has gained increasing support. Many of those who contributed to the development of this model have been closely associated with Nathan Shock at the Gerontology Research Center, including: William H. Adler, Gerontology Research Center; Julia W. Albright, George Washington University; John J. Farrar, Hoffman-La Roche; Margaret L. Heidrick, University of Nebraska; Toshio Hirano, Osaka University; Katsuiku Kirokawa, Tokyo Metropolitan Institute of Gerontology; Marguerite M.B. Kay, Texas A&M University; Hideo Nariuchi, Tokyo University; Albert A. Nordin, Gerontology Research Center; and George S. Roth, Gerontology Research Center. It would be fitting, therefore, to honor his 80th birthday by describing the T cell model of aging.

We will begin by defining the T cell lineage and commenting on the advantages of the T cell model. This will be followed by a discussion of the changes in the penultimately differentiated, antigen/mitogen responsive T cells and in their precursors. We will close by commenting on the possible types of studies which can be anticipated.

T CELL LINEAGE

At least seven stages contribute to T cell ontogeny (Keller and Phillips, 1982; Rosse, 1976; Stutman, 1978): (a) pluripotent lympho-hematopoietic stem cell, (b) lymphoid-

Correspondence to: Takashi Makinodan, Ph.D., Director, GRECC 691/W11G, VAMC West Los Angeles, Los Angeles, CA 90073.
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TABLE 1. T CELL LINEAGE

Stages	Tissue Site	Age-related alterations[1]
I. Pluripotent Stem Cell	Bone Marrow	Yes
II. Oligopotent Stem Cell	Bone Marrow	?
III. Unipotent Prethymic Stem Cell	Bone Marrow	Yes
IV. Intrathymic T Cell	Thymus	Yes
V. Postthymic T Cell	Spleen, Lymph Nodes	?
VI. Antigen/Mitogen Responsive T Cell	Spleen, Lymph Nodes, Circulation	Yes
VII. Effector T Cell	Spleen, Lymph Nodes, Inflammatory Sites	Yes

[1]Alterations could be quantitative and/or qualitative.

committed oligopotent stem cell, (c) unipotent prethymic stem cell, (d) intrathymic T cell, (e) postthymic T cell, (f) antigen/mitogen responsive T cell, and (g) effector T cell (Table 1).

There are certain unique characteristics of the T cell lineage. For instance, most of the T cells generated in the intrathymic stage die (McPhee *et al.*, 1979; Scollay and Shortman, 1983). Another unique characteristic is that many of the penultimately differentiated antigen/mitogen responsive T cells are mitotically quiescent, similar to the pluripotent lympho-hematopoietic stem cells, and thus are resistant to mitotic poisons over an extended period of time. For example, we found that 40% of murine splenic T cells are mitotically inactive, as judged by their resistance to continuous exposure to 6-thioguanine (Akagawa *et al.*, 1984).

ADVANTAGES OF THE T CELL MODEL OF AGING

In view of the recent technological advances in somatic cell genetics, it would be timely to investigate the genetic basis of the influence of aging on the differentiation process. The T cell model has been selected by many investigators as their model of choice for various reasons, including the following:

a. Alterations in age-related, T cell-dependent immunologic activities in humans are comparable to those in experimental animals, especially mice (Makinodan and Yunis, 1978).
b. These alterations are associated with specific morphologic and metabolic changes in the T cells (Makinodan, 1983).
c. Large numbers of T cells can be sampled with relative ease, and age-related T cells can be subjected to genetic analysis by clonal selection (Albertini *et al.*, 1982).
d. Identification of the stages of T cell differentiation that are vulnerable to genetic alteration is possible because much is known about their lineage, as discussed above.

AGE-RELATED CHANGES IN THE PENULTIMATELY DIFFERENTIATED, ANTIGEN/MITOGEN RESPONSIVE T CELLS

Alterations in T cell-dependent immune functions with age can result from (a) changes in the number of T cells, (b) an imbalance in the enhancing and suppressive activities of regulator cells, and (c) qualitative changes in the T cells. All three types of change have been observed (Doggett *et al.*, 1981).

Quantitative Changes

In humans it was found via the use of monoclonal antibody reagents that the total number of circulating lymphocytes decreases with age by about 15%, due primarily to a decrease in the number of T cells (Ligthart *et al.*, 1985; Nagel *et al.*, 1983; O'Leary *et al.*, 1983). In mice the pattern and magnitude of change in the number of T cells was found to vary with the tissue and organ source; in general, the number does not change appreciably with age. Thus, cell loss does not appear to contribute significantly to the changes in T cell-dependent immune functions with age.

Imbalance in Regulatory Cells

In recent years, subpopulations of T cells with helper and suppressor cell markers have been assessed in aging humans and mice with greater accuracy through the use of monoclonal antibody reagents. In humans both subpopulations decrease to about the same extent with age and, therefore, their proportion is not affected by aging (Ligthart *et al.*, 1985; Nagel *et al.*, 1983; O'Leary *et al.*, 1983). Similarly, in mice the total number and proportion of T cells with helper and suppressor cell markers are not affected by age (Chang *et al.*, 1982; Kay *et al.*, 1979; Thoman and Weigel, 1981).

Functionally, both decreases and increases in T helper and T suppressor activities have been observed. An example of a decrease in T helper cell activity is the decline in interleukin-2 (IL-2) production (Chang *et al.*, 1982; Gillis *et al.*, 1981; Gilman *et al.*, 1982; Miller and Stutman, 1981; Thoman *et al.*, 1981). An example of an increase in activity is the demonstration that T helper cells of old humans can provide more help for B cells to produce immunoglobulins than those of young humans (Ceuppens, 1982). Similarly, decreases and increases in T suppressor cell activities have been observed, depending upon the immunoglobulin index, status of stimulation, antigenic specificity, species, strains within species, etc. (Makinodan and Adler, 1975). Investigations of the regulator monocytes/macrophages show that the suppressor activity is elevated in old humans (Antonaci *et al.*, 1983; Delfraissy *et al.*, 1982) and old mice (Globerson *et al.*, 1981; Rosenstein and Strausser, 1980). The enhanced suppressor activity may be related to an increase with age in prostaglandin sensitivity (Goodwin, 1982) and/or production (Bash, 1983; Delfraissy *et al.*, 1982). In regard to the immunoenhancing activity of monocytes/macrophages, it was found that the capacity of macrophages to synthesize interleukin-1 (IL-1), a cytokine required for T cell proliferation and differentiation, is reduced in mice (Bruley-Rosset and Vergon, 1984; Inamizu *et al.*, 1985), unlike that in rats which showed no change with age (Rosenberg *et al.*, 1983). Of interest, the decrease in IL-1 production with age in mice was shown to be caused by alteration in both the macrophages and the participating T cells (Inamizu *et al.*, 1985).

Although often contradictory, these results suggest that a shift in the regulatory activities of T cells and monocytes/macrophages is occurring with age. However, the shift could reflect qualitative changes as well, at least in the case of certain T cell populations. This suspicion stems from the demonstration that the proportion of T cells with enhancing and suppressor markers does not change appreciably with age as discussed earlier and that immune cells undergo qualitative changes as will be discussed.

Qualitative Changes

The evidence for qualitative changes in immune cells derived from metabolic, morphologic, and genetic studies is most impressive.

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Changes at the Surface Membrane Level. At the surface membrane level, significantly fewer IL-2 receptors have been detected in antigen/mitogen-activated T blast cells from old animals than from young. This was demonstrated in humans (Gillis *et al.*, 1981), rats (Gillis *et al.*, 1982), and mice (Chang *et al.*, 1982), as judged by the ability of activated T blast cells to absorb IL-2. It appears that the reduction in the number of IL-2 receptors with age is contributing to the age-related decline in the proliferative activity of T cells because activated T cells require IL-2 to proliferate. In addition, the synthesis of IL-2 is also diminished with age (Chang *et al.*, 1982; Gillis *et al.*, 1981; Gilman *et al.*, 1982; Miller and Stutman, 1981; Thoman and Weigle, 1981).

A decrease in the number of glucocorticoid and beta-adrenergic receptors has also been reported in T cells of old humans (Shocken and Roth, 1977) and rats (Petrovic and Markovic, 1975), but the findings on the reduced number of beta-adrenergic receptors in old humans could not be confirmed by others (Abrass and Scarpace, 1981; Landmann *et al.*, 1981). However, it was found that T cells of old humans with intact beta-adrenergic receptors have reduced adenylate cyclase activity; this defect appears to be in the catalytic site rather than the nucleotide coupling site of the enzyme complex (Abrass and Scarpace, 1982). Finally, a change in membrane fluidity in T cells of old humans has been observed (Noronha *et al.*, 1980).

Changes at the Cytoplasmic Level. Morphologically, swollen mitochondria containing myelin-like structures with reduced numbers of cristae have been observed in the cytoplasm of T cells of old but not young humans (Biro and Beregi, 1979). Such a morphologic change, coupled with certain metabolic changes detected in cells of old but not young individuals suggests that the ability of antigen/mitogen-stimulated old cells to generate energy may be impaired.

Metabolically, a variety of age-related changes have been detected at the cytoplasmic level. Alteration in Ca^{++} uptake in mitogen-stimulated T cells of humans (Kennes *et al.*, 1981) and in the levels of cyclic adenosine monophosphate and guanosine monophosphate of unstimulated and mitogen-stimulated T cells of old mice (Tam and Walford, 1978) and humans (Tam and Walford, 1980; Birkenfeld and Ben-Zvi, 1984; Halper *et al.*, 1984) suggests that the second messenger activities may also be altered in old individuals. A decrease has also been reported in the level of nicotinamide adenosine dinucleotide (NAD) (Chapman *et al.*, 1983) and adenoside triphosphate (ATP) (Verity *et al.*, 1983) in mitogen-stimulated T cells of old humans. These metabolic changes, together with an accumulation of triosephosphate isomerase (Tollefsbol and Cohen, 1984), suggest that glycolysis may also be impaired, especially since it was previously demonstrated that glycolytic but not oxidative metabolism is essential for blast cell transformation (Roos and Loos, 1970). A decrease in the NAD level of mitogen-stimulated T cells also suggest that the ability to repair DNA could be altered. This suspicion is based on the observation that NAD can serve as a substrate for the synthesis of poly(adenosine diphosphate-ribose) which is involved in unscheduled DNA synthesis (Berger and Sikorski, 1980; Sims *et al.*, 1981).

Changes at the nuclear level. A variety of morphologic alterations with age have been observed at the level of the nucleus. The loss of chromosomes was first of these changes to be detected, with X and Y chromosomes being lost most frequently (Jacobs *et al.*, 1961). This suggests that loss of important genes in these chromosomes, especially those engaged

in regulatory functions, could play an important role in aging. A decrease in the nuclear organizing region (Buys *et al.*, 1979) has also been detected, which indicates that a decrease in the ribosomal DNA content and/or the transcriptional activity of rDNA (Zakharov *et al.*, 1982) may be occurring in T cells of aging individuals. A decrease in the binding of actinomycin by mitogen-stimulated T blast cells has been observed (Preumont *et al.*, 1978), which may reflect structural alterations in the chromatins. More recently, an increase in the frequency of T cells with micronuclei has been observed (Norman *et al.*, 1984), which could be contributing to the subpopulation of T cells which do not proliferate in response to antigenic or mitogenic stimulation.

Several functional changes have been detected in the nucleus of peripheral T cells of old individuals including (a) a decrease in the ability of T cells to repair ultraviolet light-induced damages (Lambert *et al.*, 1979; Neidermuller, 1982); (b) a decrease in the level of low molecular weight DNA polymerase, which presumably is involved in DNA repair (Barton and Yang, 1975); (c) presence of extra-chromosomal inter-Alu DNA circles in certain T cells (Yamagishi *et al.*, 1985), suggesting that transpositional events may occur with age; (d) a decrease in the activity of purine nucleotide phosphorylase, a purine salvage enzyme whose absence can cause the level of toxic deoxyribonucleoside to increase (Scholar *et al.*, 1980); and (e) a possible defect in the ability of the nucleus of mitogen-stimulated T cells to respond to the cytoplasmic stimulatory factors (Gutowski *et al.*, 1984). Finally, an age-related increase in the proportion of T cells which can proliferate in the presence of 6-thioguanine has been observed in humans (Trainor *et al.*, 1984; Vijayalaxmi and Evans, 1984) and mice (Inamizu *et al.* 1986). The fact that these 6-thioguanine-resistant T cells possess levels of hypoxanthine guanine phosphoribosyl transferase (HGPRT) activity as low as those of HGPRT-deficient mutant cells and cannot grow in HAT (hypoxanthine-aminopterin-thymidine) medium suggests that these metabolic changes are reflective of changes at the genomic level. The studies of individual aging mice further show that an inverse correlation exists between the frequency of 6-thioguanine resistant T cells and the ability of T cells to produce IL-2 or proliferate in response to mitogenic stimulation (Inamizu *et al.*, in press).

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It is clear that functional and/or morphologic changes can be detected in antigen/mitogen-responsive T cells. It would appear that many of the changes found at the nuclear level of these T cells are genetic in origin. The changes at the cytoplasmic and surface membrane levels, on the other hand, could be either nongenetic or genetic. If the changes are nongenetic and caused by extrinsic factors in the systemic milieu, they could be reversed through dietary manipulation. However, if the changes are genetic in origin, reversal may not be possible. Moreover, the changes detected in the antigen/mitogen-responsive T cells could have occurred earlier in their differentiation process. This suspicion is based on the fact that (a) a significant proportion of the antigen/mitogen-responsive T cells are mitotically quiescent, as demonstrated by their resistance to 6-thioguanine (Akagawa *et al.*, 1984); and (b) mitotically active cells are highly receptive to genetic alterations, as readily demonstrated in rapidly dividing tissue culture cells (Chu, 1974).

Age-related Changes in Bone Marrow Cells

The only direct study which has examined the influence of age on mitotically quiescent, pluripotent stem cells was reported recently by Akagawa *et al.* (1984). Young and old BALB/c mice were subjected to continuous exposure to 6-thioguanine, which selectively kills normal mitotically active cells while sparing mitotically quiescent cells. When the number of stem cells which were resistant to 6-thioguanine was determined, old mice were found to possess seven times more 6-thioguanine-resistant stem cells than young mice. They then showed that these 6-thioguanine-resistant stem cells can be induced to become mitotically active colony-forming stem cells which are then susceptible to 6-thioguanine killing. These results suggest that regulatory stromal cells, which are involved in stimulating mitotically quiescent stem cells to differentiate into mitotically active oligopotent stem cells, may have undergone changes with age.

The experimental studies on the influence of age on oligopotent stem cells have used a heterogeneous population of pluripotent and oligopotent stem cells because there is no practical method of separating them. The results of experiments using mixed stem cells show that some but not all parameters are resistant to aging. The parameters which are resistant to aging include the total number of stem cells (Chen, 1971), the ability of stem cells to differentiate into blood cells and immune cells (Harrison *et al.*, 1977), and the self-renewal ability of stem cells (Ross *et al.*, 1982). One of the parameters which is vulnerable to aging is the ability to repair ionizing radiation-induced DNA damage (Chen, 1974). Contradictory results have also been obtained from these mixed stem cell studies, i.e. results indicating a decrease (Albright and Makinodan, 1976; Lipschitz and Udupa, 1984) or no change (Harrison *et al.*, 1978) with age in the ability to stem cells to expand clonally in vivo and to generate progeny cells in vitro. In contrast, studies on unipotent prethymic stem cells have been less controversial. The results suggest that the unipotent stem cells are more vulnerable to aging than oligopotent stem cells. This is reflected by (a) a decrease with age in the ability of bone marrow stem cells to migrate into the thymus of irradiated mice (Tyan, 1977), (b) the cessation of stem cells of old mice migrating into the thymus when old mice are parabiosed to young mice (Kay, 1984), (c) a decrease in the number of bone marrow stem cells which are responsive to ubiquitin, a thymic factor (Twomey *et al.*, 1982), and (d) a decrease in the repertoire of alloreactive cytolytic T cells in the spleen and thymus of irradiated mice transplanted with bone marrow cells of old donors (Gorczynski and Chang, 1984).

Resolution of the contradictory reports on the influence of age on oligopotent stem cells would seem possible. This optimism is based on the above findings indicating that the less differentiated, oligopotent stem cells are more resistant to aging than the more differentiated, unipotent stem cells. One approach is to resolve whether the proportion of the more differentiated stem cells to the less differentiated stem cells in the test bone marrow inoculum differed between the investigators. Another approach is to resolve whether the short-term assays [e.g. the splenic colony-forming assay employed by Albright and Makinodan (1976)] detect the more differentiated stem cells and the long-term repopulation assays of Ross *et al.* (1982) and Harrison *et al.* (1982) detect the less differentiated stem cells. Other approaches are to resolve whether differences exist in the stem cell composition and disease status of the old test animals used by the different investigators.

In contrast to the effort expended in assessing the influence of age on parenchymal stem cells, studies on stromal cells have been minimal. Moreover, with few exceptions, assessment

of stromal cell activity has been indirect, which could be responsible for the contradictory results. Observations which tend to support the view that stromal cells are resistant to aging include: (a) the self-renewing ability to stromal cells of old mice is comparable to that of young mice (Mauch *et al.*, 1982), and (b) the marrow of old and young mice mice were repopulated after radiation ablation (Schofield *et al.*, 1985). Results supporting the view that stromal cells are susceptible to aging include the observations that (a) the proliferative capacity in vitro of bone marrow cells declines with age (Lipschitz and Udupa, 1984; Mauch *et al.*, 1982), (b) the ability to resynthesize stem cell regulator factors decreases with age (Schofield *et al.*, 1985), (c) the number of mitotically quiescent pluripotent stem cells increases with age (Akagawa *et al.*, 1984), and (d) the bone marrow of old but not young mice produces suppressor factors (Astle and Harrison, 1984). On the other hand, observations that stormal cells of old mice produce in vitro a greater total cellularity and a greater number of splenic colony-forming stem cells than those of young mice (Mathews and Crouse, 1981) support the view that aging enhances the activity of stromal cells. These contrasting results clearly emphasize the need for direct functional assessment of individual stormal cells.

Age-related Changes in the Thymus

The thymus is involved in four important functions: (a) the production of chemotactic factors to attract prethymic stem cells to the gland (Pyke and Bach, 1979); (b) the induction of self tolerance and major histocompatibility complex (MHC) restriction (Zinkernagel and Doherty, 1979); (c) the production of factors that act on intrathymic immature T cells, influence the peripheralization of T cells, and modulate postthymic immature T cells (Pyke and Bach, 1979); and (d) the production of factors that modulate the neuroendocrine circuit (Hall and Goldstein, 1981). To date, four thymic peptides with hormonal activities have been chemically defined: thymic humoral factor (Trainin *et al.*, 1975), thymopoietin (Schlesinger and Goldstein, 1975, thymosin α_1 (Goldstein *et al.*, 1977), and thymulin (Dardenne *et al.*, 1977). These hormones are found in the thymic epithelial cells (Haynes *et al.*, 1983; Hirokawa *et al.*, 1982) and therefore are presumed to be synthesized by them. However, very little is known about what role other stromal cells (fibroblasts, macrophages, and dendritic cells) play in the differentiation and regulation of the parenchymal T cells.

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The thymus starts to involute at sexual maturity in humans (Boyd, 1932) and mice (Santisteban, 1960), which coincides with the cessation of prethymic stem cell migration into the thymus as judged by parabiosis of mice of different ages (Kay, 1984), and with the onset of age-related decline in T cell-dependent antibody response (Makinodan and Peterson, 1962). This suggests that thymic involution contributes to the decline in T cell-dependent immunity with age. Evidence in support of this view is substantial. One example is that adult thymectomy of autoimmune-susceptible and autoimmune-resistant mice accelerates the decrease in T cell-dependent humoral and cell-mediated immune responses (Metcalf, 1965; Taylor, 1965), increases autoimmunity (Teague *et al.*, 1970), and decreases longevity (Jeejeebhoy, 1971).

Immunohistologic studies by Hirokawa *et al.* (1982) of thymic tissues from one-day to 62-year-old humans established that thymosin α_1, a representative thymic hormone, is found in the epithelial cells of the cortical surface and the medulla, and that aging selectively affects those in the medulla. Thus, they found that the number of thymosin α_1-containing epithelial cells in the medulla decreases progressively beginning at sexual maturity, so that

only a fraction can be detected by the third decade of life. In contrast, those covering the cortical surface could still be detected in adequate numbers even in the atrophic thymus of a 62-year-old individual. In regard to thymosin α_1-containing epithelial cells, it is interesting to note that Haynes *et al.* (1983) found that they also contain thymopoietin and the human T cell leukemia virus antigen p19.

In order to understand better the functional changes occurring in the involuting thymus, we assessed the capacity of involuting thymic grafts to generate antigen/mitogen responsive mature T cells (Hirokawa and Makinodan, 1975; Hirokawa *et al.*, 1982). This was done by grafting thymic lobes from donor mice ranging in age from 1 day to 33 months into either (a) thymectomized, x-irradiated, and bone-marrow reconstituted T cell deficient adult mice or (b) naturally athymic T cell-deficient adult mice. Both of these mouse models are deficient in T cells but rich in B cells, macrophages, null cells, precursor stem cells, and stromal cells. At intervals for a period of 12 weeks after grafting, both groups of recipient mice were assessed for various T cell dependent immunologic activities; comparable results were obtained which showed that T cell activities are inversely related to the age of the donor mice (i.e. the older the donor mice, the lower the T cell activity), but the degree of the inverse relationship differed with the T cell activity index. For example, it appears that the ability of thymic grafts to generate cytolytic T cells against allogeneic target cells declines rapidly starting at birth, long before the thymus reaches its maximum size. On the other hand, the ability of thymic grafts to generate mitogen-sensitive T cells decreases only slightly with age, with the decline beginning at midlife, long after the start of the involuting process. These results suggest that the intrathymic and postthymic immature T cells could be the major, if not the sole progenitor source of antigen/mitogen responsive T cells after adulthood. If so, the thymus can be looked upon as the pacemaker of aging of the T cell arm of the immune system.

To gain a more precise understanding of how the thymus is aging physiologically, a series of studies were initiated to characterize the influence of age on the stromal cells (Sato *et al.*, 1984). Plastic-adhering stromal cells (which include epithelial cells, macrophages, dendritic cells, and fibroblasts) participate in the differentiation and proliferation of parenchymal thymocytes by producing either the differentiation factors, proliferation factors or their modulators. Thymic adherent cells, obtained from mice ranging in age from 1 day to 20 months, were cultured for one month in vitro with weekly supernatant collection. The supernatants were then assessed for their ability to augment or inhibit the antigen/mitogen induced proliferation of indicator thymocytes and of indicator prethymic splenic cells obtained from syngeneic nude mice. The results revealed that intrathymic but not prethymic T indicator cells are responsive to the modulating influence of the supernatant. It is therefore likely that the prethymic stem cells require a direct contact with the adherent cells in order to undergo differentiation. The results further showed that the ability of thymic adherent cells to synthesize the augmenting factor declines drastically between 2.5 and 5 months of age, which is consistent with the findings derived from histologic and thymic graft studies (Hirokawa and Makinodan, 1975; Hirokawa *et al.*, 1982). Perhaps the most interesting findings in terms of homeostasis are those indicating that the thymic adherent cells can also synthesize an inhibitory factor. Thus, thymic adherent cells of newborn mice were shown to synthesize both the augmenting factor and the inhibitory factor, while those of young adult mice synthesize primarily the augmenting factor, and those of 20-month-old mice synthesize primarily the inhibitory factor. Mixture studies of the 2 fac-

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tors suggest that these factors can coexist and that the augmentable thymocytes are the target of the inhibitory and augmenting factors.

Our current effort has been centered on the inhibitory factor because it is produced primarily by the involuted thymus of aging mice (Kinohara *et al.*, 1985). Our initial study showed that the inhibitory factor suppresses the proliferation of crude interleukin 2 stimulated, immature and mature thymocytes, but not of crude IL-2 stimulated T blast cells nor of crude IL-2 stimulated cytolytic T cells (CTLL-2). These results suggest that the inhibitory activity of the factor could be T cell differentiation stage-specific. Our gel filtration analysis, using ultrogel AcA54, then showed the inhibitory activity in the supernatant of thymic adherent cells of young mice was found mainly in the 68,000 fraction, whereas that of the involuting thymus of aging mice was polydispersed. The latter supernatant was therefore subjected to DEAE ion exchange chromatography and, among the three fractions containing activity, the fraction with the highest inhibitory activity was then subjected with Sephacryl S-300 gel filtration. The results showed that the activity was confined to the 68,000 fraction, which contains serum albumin. However, albumin itself had no inhibitory activity. Moreover, the inhibitory activity was heat-resistant (75°C/30 minutes).

Further studies are required to (a) characterize the physical and biochemical properties of the factors that inhibit or augment thymocyte proliferation; (b) identify the specific thymic adherent cells that are synthesizing the inhibiting and augmenting factors; and (c) determine the molecular basis for the synthesis of these proliferation modulating factors.

CONCLUDING REMARKS

An attempt has been made to summarize our current knowledge of the vulnerability of T cells to aging. Of the three possible changes which are associated with altered T cell-dependent immune functions (loss of T cells, imbalance of regulatory cells, and qualitative changes in T cells), qualitative cellular changes are the most prominent, and those occurring at the nuclear level are the most dramatic. This suggests that there is a genetic basis for at least some of the changes.

If the phenotypic changes observed in the antigen/mitogen-responsive T cells are genetic and can be transmitted to progeny effector cells, then an important issue would be to resolve where in the T cell lineage these changes originate. The results of current studies suggest that they could occur in the bone marrow, thymus and/or postthymic stages (Table 1). A picture is emerging which suggests that many control points may exist along the path of gene expression (Alberts *et al.*, 1983) which could be vulnerable to aging. Thus, gene alterations caused by gene diminution, amplification, rearrangement, or modification, and modulation of gene expression at the transcriptional, post-transcriptional or translational level should be taken into consideration.

Based on these considerations, efforts will be expanded to (a) differentiate genetic from nongenetic changes, (b) pinpoint where in the T cell lineage stable genetic changes occur, and (c) identify the control points in the expression of important T cell functions which are affected by aging.

The T cell system will continue to serve as an important model for the study of how aging modulates the cellular differentiation process, especially at the molecular and genetic levels. It should be noted that Nathan Shock played a crucial role during the formative stages 15 years ago, for as Director of Gerontology Research Center, he fostered the research which led to the development of T-cell model of aging.

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IMPAIRED CONVERSION OF RAT UTERINE ESTRADIOL RECEPTORS DURING AGING

RUMIANA S. CHUKNYISKA, CYNTHIA JUSTINIANO and GEORGE S. ROTH

Molecular Physiology and Genetics Section, Laboratory of Cellular and Molecular Biology, Gerontology Research Center, National Institute on Aging, Francis Scott Key Medical Center, Baltimore, Maryland 21224

Abstract—We have examined the effects of aging on the capacity of rat uterine estradiol receptors to be transformed from 8S to 4S and 5S species. Cytosol receptors from mature (6-month-old) rats or senescent (24-month-old) rats have been exposed to various KCl concentrations, ammonium sulfate precipitation and 25°C heating. Estradiol receptors of both the mature and senescent age groups exist in an 8S form on linear 5–20% sucrose gradients in the absence of KCl and are converted to a 4S molecule in the presence of 0.4 M KCl. At intermediate salt concentrations a greater portion of mature receptors was converted to the 4S species. At 0.15 M KCl 62.3% ± 2.8 of the mature receptors are converted to 4S versus 41% ± 1.9 of the senescent receptors, and at 0.2 M KCl 79.6% ± 3.2 of the mature receptors are converted to the 4S versus 58.2% ± 2.1 of the senescent. Ammonium sulfate treatment in the presence of 0.3 M KCl converted about 80% of the receptors from the 4S to the 5S form, while only about half of the old receptors are affected. When ammonium sulfate precipitates were heated to 25°C all to mature receptors were converted to the 5S species, while only two thirds of the senescent receptors were sedimented at 5S under the same conditions. Inclusion of 20 mM molybdate during preparation blocks conversion of about 15% of the senescent receptors from the 8S to the 4S form but does not affect the mature preparations. Simlarly, molybdate treatment does not affect the conversion of the mature estradiol receptors to the 5S form but increases the percentage of senescent receptors remaining in the 4S form from 30 to 45%. Such qualitative differences in receptor conversion may be related to age associated deterioration of estradiol stimulated uterine responsiveness.

Key Words: aging, estradiol receptors, conversion, uterus

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INTRODUCTION

THE ABILITY of estradiol to increase rat uterine RNA polymerase II activity in vivo and in vitro declines during aging (Haji *et al.*, 1984; Haji and Roth, 1984) and may be responsible for various deficiencies in stimulated gene expression (Stone *et al.*, 1977; Jensen and DeSombre, 1972). Such age and associated deficits are due to both loss of uterine estradiol receptors (Gessel and Roth, 1981; Saududdin and Zassenhaus, 1979; Nelson *et al.*, 1976; Hseueh *et al.*, 1979), and impaired association of receptor estradiol complexes with nuclei

Correspondence to: Dr. Rumiana Chuknyiska, Gerontology Research Center, National Institute of Aging, NIH, 4940 Eastern Avenue, Baltimore, MD 21224.
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(Tang and Peng, 1981; Belisle and Lehoux, 1982; Belisle *et al.*, 1983; Chuknyiska *et al.*, 1985; Chuknyiska and Roth, 1985). The latter dysfunction, in turn, seems to be a consequence of age related deterioration of both receptors and nuclei (Chuknyiska *et al.*, 1985). In an initial attempt to further elucidate these changes, we have examined the effects of aging on the capacity to convert rat uterine estradiol receptors from 8S to 4S and 5S, prerequisites for nuclear binding (DeSombre *et al.*, 1972; Notides and Sasson, 1983; Weichman and Notides, 1979).

Although recent evidence suggests that estradiol receptors may normally be found in association with the nucleus (Welshons *et al.*, 1984) it is clear that much tighter binding occurs after physiochemical changes that alter receptor sedimentation properties from 8S to a state of high affinity (Müller *et al.*, 1977; Giannopoulos and Gorski, 1971). Such changes can be elicited in vitro by salt treatment, dilution or heating (Jensen and DeSombre, 1972; Notides and Nielsen, 1974; Müller *et al.*, 1977). In the present study, receptors have been exposed to similar conditions (i.e. KCl at various concentrations, ammonium sulfate precipitation, and heating to 25°C).

Our earlier studies which revealed age related reductions in estradiol stimulated RNA polymerase II and nuclear binding of receptor-estradiol complexes (Haji *et al.*, 1984) were carried out under conditions of 25°C exposure, 35% ammonium sulfate precipitation and 0.3 M KCl contining buffer. Under all of these conditions, senescent preparations were found to be less capable of being converted to the 4S and 5S forms (Chuknyiska and Roth, 1985). Such qualitative charge in receptor conversion may be related to age associated deterioration of estradiol stimulated uterine responsiveness.

MATERIALS AND METHODS

Animals

Female Wistar rats 6–8 months of age (mature) and 24–25 months of age (senescent) were obtained from the Gerontology Research Center Animal Colony. This Colony is fully accredited by the American Association for Accreditation of Laboratory Animal Care. The older animals are generally in good health, without detectable uterine tumors. The only observable uterine pathology is cystitis in less than 5% of the senescent rats, and these animals are routinely discarded. The animals were bilaterally ovariectomized 1 week before cytosol receptor preparation.

Chemicals

[2, 4, 6, 7, 16, 17-^{3}H]Estradiol (151 Ci/μmol) was purchased from New England Nuclear (Boston, MA) and nonlabelled 17β-estradiol was from Sigma Chemical Co. (St. Louis, Mo). Yeast Alcohol Dehydrogenase, Albumin and Bovine Serum Albumin were purchased from Boeringer (Mannheim, West Germany). All reagents were reagent grade obtained from commercial sources. Aqueous counting scintilant from Amersham was used for radioactivity measurement.

Uterine cytosol preparation and estradiol receptor complex formation

50mM Tris-HCl, 10 mM EDTA, 12 mM monothioglycerol pH 7.4 was used routinely for isolation of uterine cytosol as previously described (Chuknyiska *et al.*, 1985). Various

concentrations of KCl were included in the isolation buffers depending upon the experimental design. Cytosols were prepared in buffer without KCl and then incubated with [^{3}H]estradiol and sufficient KCl to yield the desired final concentration. Gradient profiles were identical to those obtained when cytosols were isolated in the presence of the same KCl concentrations, in agreement with a previous report (Giannopoulos and Gorski, 1971).

Uteri were minced and homogenized in 1.5 ml of Tris-HCl buffer/g tissue with a Polytron as previously described (Haji *et al.*, 1984; Chuknyiska *et al.*, 1985). 105,000 g supernatants were separated from pellets after 1 h centrifugation and incubated with 30 nM [^{3}H]estradiol for at least 4 h in the cold room. Parallel samples were incubated for the same time with 30 nM [^{3}H]extradiol and 100-fold excess of nonlabelled estradiol to correct fo nonspecific binding. All operations were performed at 4°C unless otherwise indicated. Unbound estradiol was removed at the end of the incubation period by treatment with 0.5% charcoal and 0.05% dextran for 15 min on ice with intermittent blending on vortex followed by centrifugation at 3000 g for 10 min.

Ammonium sulfate precipitation

After incubation with [^{3}H]estradiol portions of cytosols were subjected to ammonium sulfate precipitation. Ammonium sulfate was added gradually to a final concentration of 35%, while the samples were kept on ice with constant stirring for 1 h. Precipitates were collected after 15 min by 11000 × rpm centrifugation and kept at (−70°C) until use, if necessary. Before layering on gradients, precipitate were redissolved in the corresponding buffer to 1/5 of the original cytosol volume and treated with dextran coated charcoal.

Sedimentation analysis

4.2 ml linear (5–20%) sucrose gradients were formed in 50 mM Tris-HCl at pH 7.5 with various KCl concentrations using a two chamber gradient maker from Hueffer Scientific Instruments or by hand layering of 5%, 7.5%, 10%, 12.5%, 15%, 17.5% and 20% sucrose solutions prepared in advance in appropriate buffer. Gradients were kept in ice for at least 6 h before use. Cytosol samples were adjusted for protein concentrations (equal amounts of protein from young and old uteri were applied). Gradients were centrifuged at 45000 rpm for 18 h in Beckman SW-60 rotor or at 38000 rpm for 6 h and 50000 rpm for 2 hr in a TV-865 vertical rotor. Tubes were punched at the bottom and 27 or 28 fractions of approximately 160 μl each were collected. Receptor sedimentation coefficients were determined according to Martin and Ames (1961) by using yeast alcohol dehydrogenase (7.4S), bovine serum albumin (4.6S) and ovalbumin (3.4S) as external standards. Sedimentation coefficients of uterine cytosol receptors were 8.1S under low salt conditions 3.9S under high salt conditions and 4.8S after ammonium sulfate and/or heat activation. For convenience these are referred to as 8S, 4S and 5S, respectively. For quantitative assessment of the percentage of the converted species, nonlinear curve fitting was performed using linear combination of Lorentzian functions. We assume symmetry for each peak, and one-half of the magnitude of each was calculated by integrating over the area under the left half of the left peak and the right half of the right peak. The percentage of the converted species was calculated by dividing the area of each peak by the sum of areas of both the left and right peaks.

Protein Determination

Protein concentration was measured either by the method of Lowry *et al.*, Martin and Ames 1961 or by absorbance at 280 nM on an LKB Ultraspectrophotometer 4050.

RESULTS

Effect of Age and KCl Treatment on Receptor Conversion

Since exposure to high KCl concentrations has been routinely used to convert estradiol receptors from 8S to 4S and 5S forms (Jensen and DeSombre, 1972; Müller *et al.*, 1977; Notides and Nielsen, 1979; Edros, 1968), this manipulation was initially performed on preparations obtained from mature and senescent rat uteri. Figure 1 illustrates the results of this experiment. It is clear that essentially all the receptors of both age groups exist in the 8S form on sucrose gradients in the absence of KCl, and are quantitatively converted to 4S in the presence of 0.4 M KCl. However, at intermediate salt concentrations a greater portion of mature as opposed to senescent receptors is converted to the 4S species. Figure 2 makes the point more quantitatively, indicating that 50% conversion to the 4S form occurs at about 0.12 and 0.18 M KCl for mature and senescent receptors, respectively. It should be noted that mature uteri contain higher receptor concentrations that senescent counterparts as previously reported (Gesel and Roth, 1981; Haji *et al.*, 1981). If absolute, rather than percentage, values for receptors are considered, an even lower proportion of senescent receptors exist in the converted 4S form at intermediate KCl concentrations.

Effect of age, ammonium sulfate precipitation, and 25°C heating on receptor conversion from 4S to 5S

Following 4S conversion, receptors can be shifted to a 5S species by dilution after ammonium sulfate precipitation and/or heating at 25°C (Jensen and DeSombre, 1972). Figure 3 shows the results of these treatments on mature and senescent preparations. First, it can be seen by comparison with Fig. 1 that ammonium sulfate treatment in the presence of high salt (0.3 M KCl) converts about three fourths of the mature receptors from 4S to 5S, while only about half of the senescent receptors are similarly affected. If the dissolved precipitates are heated from 0°C to 25°C, all of the mature receptors are transformed to the 5S species but only about two thirds of the senescent receptors are altered. This experiment was repeated 3 tims yielding the following values for percent conversion to the 5S form: 4°C mature−78 ± 6%, 4°C senescent−52.3 ± 4%; 25°C mature−100 ± 0%, 25°C senescent−67 ± 5%.

Effect of age and molybdate exposure on receptor conversion to 4s and to 5s Forms

Molybdate has been reported to stabilize steroid receptors effect their conversion to 4S and 5S forms (Chen *et al.*, 1981; Müller *et al.*, 1983). Figure 4 shows that inclusion of molybdate during preparation blocks the dissociation of approximately 15% of the senescent 8S receptors 4s entities but has no effect on mature preparations (B). Similarly, molybdate treatment is ineffective in preventing mature receptor conversion to 5S, but increases the percentage of senescent receptors remaining in the 4S form from approximately 30 to about 45% (D). Inclusion of molybdate in the gradients but not the preparation buffers (A and C) has no effect on either age group when compared with Fig. 1 and 3 in which molybdate was not present in the gradients.

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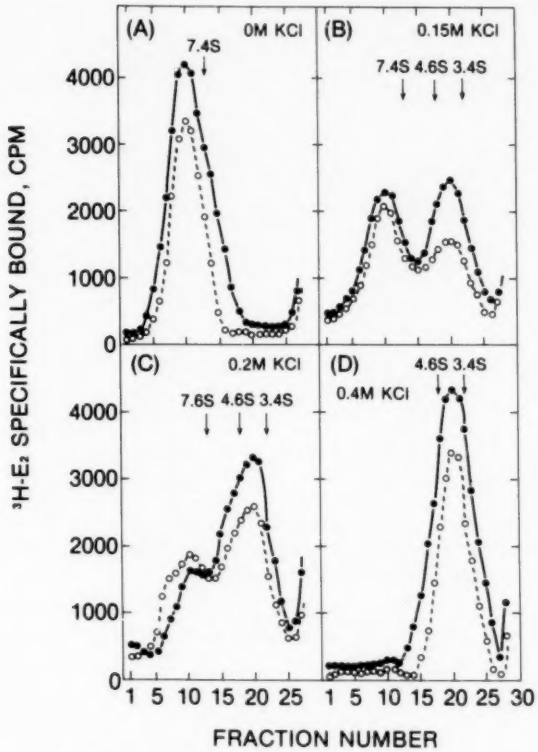

FIG. 1. Effect of age and KCl treatment on cytosol receptor sedimentation profile. Cytosol was prepared from mature and senescent uteri as described under Materials and Methods and incubated with 30 nM [^{3}H]estradiol for 4–24 h at 4°C. After dextran coated charcoal treatment, 0.2 ml of the samples containing equal protein concentration (~ 3 mg/0.2 ml) were layered on sucrose gradients containing the indicated concentrations of KCl and centrifuged and collected as described under Materials and Methods. Specifically bound cpm represent the difference between preparations incubated with [^{3}H]estradiol alone and [^{3}H]estradiol plus a 100-fold molar excess of unlabelled E_2. (●) mature; (○) senescent. Total specifically bound cpm were: (A) mature – 33,854, senescent – 23,400; (B) mature – 36,641, senescent – 26,922; (C) mature – 37,000, senescent 24,844; (D) mature – 34,069, senescent – 25,212.

Effect of mixing mature and senescent cytosols of receptor sedimentation patterns.

In order to determine whether nonreceptor components in the mature and/or senescent cytosols were influencing sedimentation patterns, mixing experiments were performed using various proportions of the respective cytosols. Table 1 shows that in the presence of 0.4M KCl and 20 mM sodium molybdate senescent receptors sediment with about 2/3 in the 4S form and 1/3 in the 8S form (see also Fig. 4B). After cytosol from mature and senescent rat uteri were obtained and cytosol receptor complexes formed in the presence of 0.4 M KCl (See Materials and Methods), portions of both preparations were incubated with 20 mM molybdate for 60 min at 4°C. Then mixtures of both cytosol receptor complexes in 1:1 or 1:2 ratios were analyzed on 5–20% sucrose gradients as described in Materials and Methods. When a mature preparation containing an equal amount of pro-

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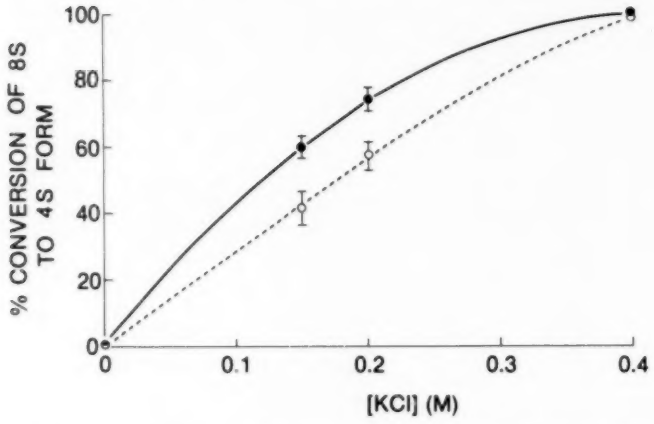

FIG. 2. Effect of KCl concentration and age on conversion of receptors from 8S to 4S form. Data from 3 separate experiments performed exactly as for Fig. 1 were obtained and the percentages of total receptors converted to the 4s form were calculated at each KCl concentration applying the method of least square fit (Wood *et al.*, 1971). Values represent the means ± standard errors. (●) Mature and (○) senescent preparations are significantly different at 0.15 and 0.2 M KCL concentrations ($p < 0.05$).

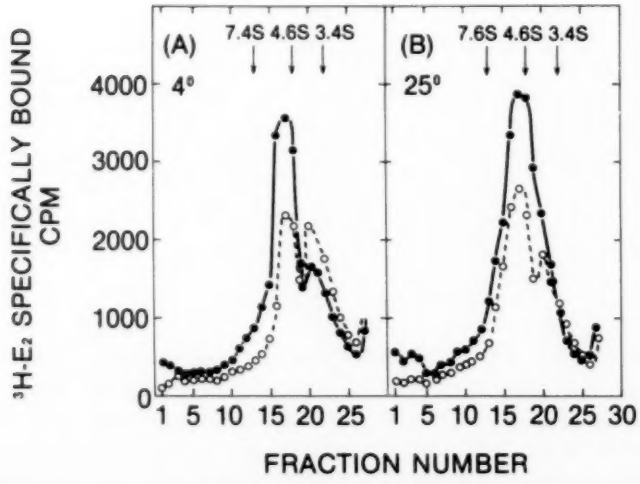

FIG. 3. Effect of ammonium sulfate precipitation and heating on receptor conversion to 5S form. Cytosols of mature and senescent uteri were precipitated with 35% ammonium sulfate, as described under Materials and Methods, and layered after dextran coated charcoal treatment on the top of 4.2 ml linear, 5–20% sucrose gradients containing 0.3 M KCl as in the isolation buffer. As indicated, all operations were carried out at 4°C with or without a subsequent exposure to 25°C for 30 min (●) mature; (○) senescent. Total specific binding—(A) mature—27,428, senescent—18,092; 25° mature—26,608; senescent—17,931.

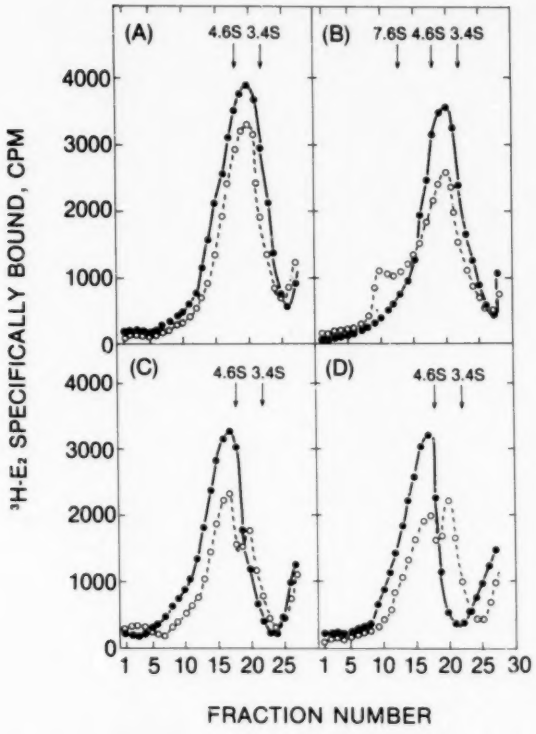

FIG. 4. Effect of age and molybdate on receptor conversion to 4S and 5S forms. Cytosol preparation and sedimentation were performed as for figs. 1 and 3, except that 20mM sodium molybdate was included in the gradients. In addition, 20mM sodium molybdate was included in preparation buffers in B and D. (○) mature; (○) senescent. A) 0.4M KCl, specifically bound cpm: mature–31,209, senescent – 23,458; B) 0.4M KCl plus 20 mM sodium molybdate during preparation, specifically bound cpm: mature–30,996; senescent – 22,341; C) 35% $(NH_4)_2SO_4$, 0.3M KCl, 25°C, specifically bound cpm: mature – 25,492, senescent – 15,557; D) 35% ammonium sulfate 0.3 M KCl, 25°C plus 20 mM sodium molybdate during preparation, specifically bound cpm: mature – 24,885, senescent – 16,100.

tein was added to a duplicate senescent preparation, the absolute amount of receptors in the 8S form was unchanged. Since essentially all of the mature receptors sediment at 4S under these conditions (Fig. 4B), neither age preparation seems to influence the other. Similarly, when a mixture composed of 2/3 senescent and 1/3 mature material was prepared under the same conditions (total protein equivalent to the 1/2 mature 1/2 senescent mixture), the amount of receptor in the 8S form remained unaltered.

Analogous results were obtained following ammonium sulfate precipitation and warming to 25°C in the presence of 20 mM sodium molybdate. In these experiments ammonium sulfate precipitates were applied to gradients following incubation of precipitate samples diluted with buffer containing 20 mM molybdate at 25°C for 30 min. Table 1 shows that under these conditions senescent receptors sediment with about 2/3 in the 5S form and 1/3 in the 4S form (see also Fig. 4D). When a mature preparation containing an equal amount of protein was added to a duplicate senescent preparation, the absolute amount

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TABLE 1. EFFECT OF MIXING MATURE AND SENESCENT RECEPTOR PREPARATIONS ON SEDIMENTATION PATTERNS

	0.4 M KCl, 20 mM sodium molybdate, 4°C			Ammonium sulfate precipitation, 20 mM sodium molybdate 25°C		
	4S	8S	Total	4S	5S	Total
Senescent cytosol (1.5 mg protein + 1.5 mg BSA)	8,422	4,182	13,604	2,285	4,931	7,216
senescent cytosol (1.5 mg protein) mature cytosol (1.5 mg protein)	27,385	4,037	31,422	2,392	15,875	18,267
senescent cytosol (2 mg protein + 1 mg BSA)	12,975	5,410	18,385	3,229	7,023	10.252
senescent cytosol (2 mg protein) + mature cytosol (1 mg protein)	24,216	5,609	29,286	3,075	14,371	17446

Cytosol receptors were prepared as for Fig. 4 B and D. Protein concentration per gradient wash adjusted to 3 mg with the desired cytosol mixture and bovine serum albumin (BSA). Values represent cpm of [^{3}H]-estradiol specifically bound in the respective peaks.

of receptors in the 4S form was unchanged. Since essentially all of the mature receptors sediment at 8S under these conditions (Fig. 4D) neither age preparation seems to influence the other. Similarly, when a mixture composed of 2/3 senescent and 1/3 mature material was prepared under the same conditions (total protein equivalent to the 1/2 mature, 1/2 senescent mixture), the amount of receptor in the 4S form remained unaltered.

DISCUSSION

Previous studies from this laboratory and others have suggested that qualitative as well as quantitative deficits in rat uterine estradiol receptors may be partially responsible for estrogen insensitivity during aging. However, most prior attempts to reveal such defects have only been successful from a functional standpoint (Nelson *et al.*, 1976; Belisle *et al.*, 1983; Belisle and Lehoux, 1982; Chuknyiska *et al.*, 1985; Chuknyiska *et al.*, 1984), and physical alterations in receptors could not readily be detected (Gesell and Roth, 1981). Receptor association and dissociation constants for both estradiol and nuclei were unaffected by age (Belisle *et al.*, 1983; Chuknyiska *et al.*, 1985) and immunochemical analyses revealed no detectable age differences (Gesell and Roth, 1981). Although one study reported that a greater portion of senescent receptors than mature sedimented at 4S under low salt conditions (Saiduddin and Zassenhaus, 1979), these results are opposite to what would be expected to account for functional decrement in receptor action in that the senescent preparation should have fewer receptors in the 4S form (Erdos, 1968; Notides and Nielsen, 1974). The present report is the first to suggest qualitative physical changes in

rat uterine estradiol receptors which might be responsible for age associated deterioration of estrogen sensitivity. Both conversion from 8S to 4S and to the 5S form are diminished in senescent receptor preparations.

Molybdate has been shown affect the conversion of steroid receptors (Shyamala and Leonard, 1980; Moudgil *et al.*, 1984; Nishigori and Toft, 1980; Müller *et al.*, 1982; Leach *et al.*, 1979). However, this effect has not been observed under high salt conditions (Müller *et al.*, 1983; Nishigori and Toft 1980; Leach *et al.*, 1979). These reported data are consistent with our observations for estrogen receptors obtained from mature uteri. One theory proposes that molybdate acts on receptor molecules through nonspecific ionic effects possibly at the same sites influenced by KCl (Mauck and Notides, 1980). If this is true, then senescent receptors may differ from mature in certain intramolecular connections, exhibiting increased lability and susceptibility to molybdate stabilization.

Conversion from 8S to 4S forms was found to be KCl concentration and time dependent (Edros 1968; Moudgil *et al.*, 1985). Our findings are in agreement with these reports and differential sensitivity with aging further suggests structural differences in the receptor molecules. Alternatively, molybdate has been suggested to act on phosphatases which destabilize receptors (Nielsen *et al.*, 1977). This, an age change in the susceptibility of these enzymes to molybdate might account for the present results.

Both 4S and 5S forms of steroid receptors have been observed in nuclei (Weichman and Notides, 1979; Notides and Nielsen, 1974; Jensen *et al.*, 1969; Musliner *et al.*, 1970) and a previous study has reported maturational changes in the amount of 4S and 5S estradiol receptor in the nuclei of the Holtzman rats (Linkie and Siiteri, 1978). It was proposed that 4S and 5S forms are in equilibrium and that the reaction follows second order kinetics (Müller *et al.*, 1984a). 4S forms of the receptor could be in either the low or high affinity state (Müller *et al.*, 1984b). A larger portion of the 4S receptor may be in low affinity state in senescent rats, thus allowing less conversion to the 5S form. This possibility is consistent with the finding that molybdate interferes with conversion only in senescent cytosol.

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The possibility that apparent deficits in the conversion of senescent receptors to the 4 and 5S forms might be due to nonreceptor factors has been at least partially eliminated by mixing experiments. Mature and senescent cytosols did not seem to influence the sedimentation patterns of each other. Thus, lack of factors necessary to stimulate conversion or the presence of factors inhibitory to conversion in senescent cytosols seems unlikely. However, until our experiments can be repeated using receptor populations purified to homogeneity, we cannot completely rule out the influence of nonreceptor components.

It is not yet known what chemical or physical changes occur in estradiol receptor molecules during the steroid binding and activation, and why old and young receptors appear to react differently. However, it has been reported that only the 5S and not the 4S receptor form activates RNA polymerase (Mohla *et al.*, 1972). Thus, impaired conversion to the 5S form might result in reduced responsiveness. It is also possible that some of 5S receptors in senescent cytosol are in a low affinity state and accordingly might not bind well to nuclear acceptor sites, thus preventing maximal estradiol action. In any case, although we cannot conclusively demonstrate that defects in receptor conversion result in impaired estrogen responsiveness, this possibility seems quite likely.

Acknowledegments—We thank Drs. E. Müller, A. Notides and V. Moudgil for their helpful discussion and advice in the preparation of this manuscript and Mrs. Rita Wolferman for its typing.

NOTE ADDED IN PROOF: Shortly after submitting these data, a report by Belisle *et al.* (Endocrinology 118: 750–750, 1986) appeared which used both heat activation and molybdate treatment of mouse uterine estrogen receptors to reach a conclusion similar to our own; *viz.* impaired receptor conversion may be responsible for decreased estrogen responsiveness during aging.

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A COLLOID OSMOTIC MODEL OF MACROMOLECULAR AGGREGATION TO EXPLAIN TISSUE WATER LOSS IN AGING

THEODORE R. REIFF

Laboratory for Nutrition and Cataract Research, Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111; Professor of Medicine, Chief Division of Geriatric Medicine, Eastern Virginia Medical School, V.A.M.C., Hampton, Virginia 23667*

Abstract—We have evolved from the sea, are composed mostly of water, the medium and solvent wherein most vital processes occur, and phylogenetically and ontogenetically lose water continually as we age.

An embryo is about 90% water, a newborn child about 80% water, a mature adult about 70% water, an older adult about 60% water with recent work indicating that in senescence the percentage of body water is actually below 60%.

The mechanisms of the water loss in aging have not been elucidated. From a theoretical point of view, there is good reason to believe that there may be profound changes in the chemical potential of intracellular and interstitial water with age due to increased macromolecular interaction or aggregation from cross linking, polymerization, insolubility, etc.; all of which are known to increase with aging. The resultant increased macromolecular solute-solute interaction would be accompanied by decreased macromolecular solute-solvent interaction, thereby causing a higher solvent (water) chemical potential. This would facilitate the loss of bound water, thereby explaining the observed losses in body water with age.

The ocular lens is a microcosm of aging, in that from its nucleus to cortex, the oldest to youngest cells are concentrically arranged, as in a tree. We have developed a method to directly measure lens tissue oncotic pressure in an attempt to experimentally test the above cited hypothesis.

Key Words: colloid osmotic mechanism, oncotic mechanism, macromolecular aggregation, tissue water loss, aging water loss, colloid osmotic pressure, oncotic pressure, body water

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INTRODUCTION

A REVIEW OF previous work indicated considerable confusion, discrepancy, and disagreement about body water changes with age. Some of the conflicting findings related to the manner in which water content was measured and how it was expressed, i.e. water/individual, water/total body mass, water/lean body mass.

The literature, i.e. (Hines and Knowlton, 1937; Steele *et al.*, 1950; Von Hevesy and

*Correspondence to this address.
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Hofer, 1934; Pale *et al.*, 1947; Berger *et al.*, 1950; Shock *et al.*, 1963 Lowery and Hastings, 1952; Cohn and Shock, 1949; Lesser and Markofskey, 1979; Lindeman 1981; Steen and Isacsson, 1985), also has conflicting conclusions with relation to the amount of water loss between intracellular and extracellular compartments. Experimental methodology has varied in the tracer utilized to measure body compartments. Although antipyrine has been utilized (Steele *et al.*, 1950; Berger *et al.*, 1950) deuterium oxide and/or tritium oxide (Von Hevesy and Hofer, 1934; Pale *et al.*, 1985) have been felt to be superior for the measurement of total body water and, from a physico-chemical point of view, would logically appear to be preferable.

Inulin, bromide, thiocyanate, etc. have been utilized to estimate extracellular field. Intracellular fluid has usually been calculated from the difference between total body water and extracellular fluid.

Although earlier studies indicated that the loss in total body water with age was mostly of the intracellular water compartment, recent work (Steen, *et al.*, 1985) has revealed significant decrements in extracellular water. While some of the change in body composition is related to the known increase in body fat there is general agreement that body water does decrease with age.

COLLOID OSMOTIC HYPOTHESIS OF SENESCENT WATER LOSS

From a theoretical point of view, there is good reason to believe that there may be profound changes in the chemical potential of intracellular and interstitial water with age, related to increasing macromolecular interaction or aggregation (crosslinking, polymerization, insolubility, etc.). The increase in macromolecular solute-solute interaction would be accompanied by a decrease in macromolecular solute-solvent interaction resulting in a higher solvent (water) chemical potential. This would allow for an easier loss of bound water with age.

The physical phemonenon responsible for water retention in cells is the oncotic pressure or colloid osmotic pressure exerted by macro molecules, largely protein molecules, within the cell. A review of some of the simple physical aspects of osmotic pressure may help elucidate and develop this thesis. Osmotic pressure can best be explained by reference to an osmometer, a device that measures osmotic pressure.

The osmometer is a container divided into two sections by a semipermeable membrane which allows solvent particles (water molecules) to pass through in either direction (Fig. 1).

The rates of movement of solvent through membrane in either direction may be represented by vectors whose length is proportional to the rate of transport of solvent from one side to the other. The algebraic sum, which is the difference between the two vectors, is the net rate of transport of solvent across the membrane. One side of the container holds pure solvent, and the other a solution of macromolecular solute in solvent. The rate of transport of solvent from the solution side is less than the rate of transport of solvent from the pure solvent side because the concentration of solvent in the solution side is less than the concentration of solvent on the pure solvent side. The rate of transport is proportional to the concentration of the material being transported, i.e., the solvent.

Accordingly, net solvent will be transported from the pure solvent side to the solution side until the differential pressure is high enough to equalize the rates of transport of solvent from one side to the other, resulting in an equilibrium.

The pressure which is necessary and sufficient to equalize the rates of solvent transport,

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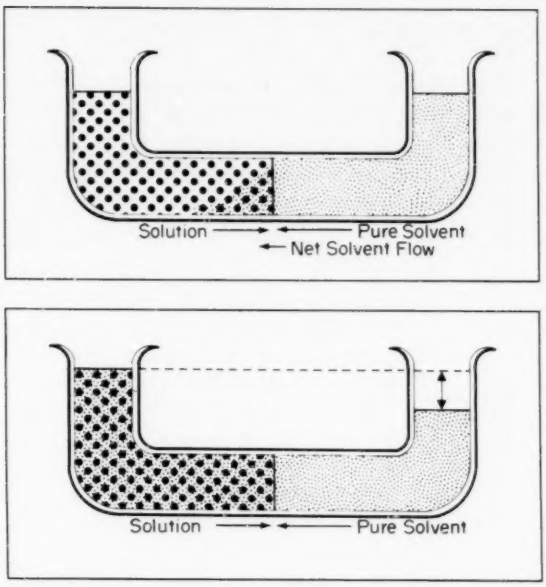

FIG. 1. Osmotic pressure concept is schematized by osmometer in which macromolecular solution is separated from pure solvent by semipermeable membrane, permeable by solvent but not by solute. Net flow of solvent will be toward solution since the chemical potential (concentration) of the solvent is lower on solution side (top). This flow of solvent will be halted by applying a sufficiently high pressure (the osmotic pressure) to the solution. Such equilibrium will be attained when enough solvent has been transported across the membrane to raise the height of the solution sufficiently to exert an osmotic pressure (bottom).

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resulting in zero net solvent transport, is the osmotic pressure. The relationship of osmotic pressure of a solution to the concentration, or number of molecules of solute per unit volume of solution, is linear for "ideal" solutions (Fig. 2).

Experimentally, it is found that for low molecular weight solutes the osmotic pressure is lower than expected. This is caused by solute-solute interaction, which, in effect, reduces the concentration of particles of solute that have partially combined with each other thereby lowering solute-solvent interaction, with a consequent reduction of the measured osmotic pressure. Solute-solute interaction can be thought of as making a negative contribution to the ideal line, resulting in a lower osmotic pressure than the ideal. For high-molecular weight solutes such as proteins, the osmotic pressure is higher than expected. This is due to increased solute-solvent interaction, which has the effect of raising the osmotic pressure as if there were a higher concentration of solute. Increased solute-solvent interaction can be thought of as adding a positive contribution to the ideal line. The increased solute-solvent interaction can be attributed to the trapping of solvent by macromolecular micelles or clusters that form at high concentrations and to Gibbs–Donnan effects of charged macromolecules that necessitate ionic imbalances causing resultant increase in solute-solvent interaction.

An alternative, and perhaps simpler way of viewing osmotic pressure is to think of two

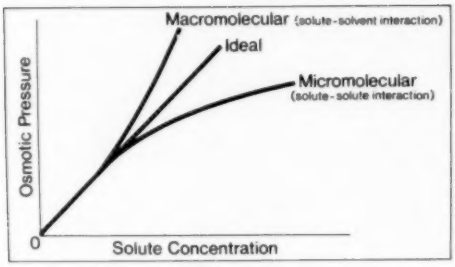

FIG. 2. Relationship of osmotic pressure and solute concentration is linear only for "ideal" solutions of intermediate-size molecules. For solutions of micromolecular particles, solute-solute interaction predominates, thereby lowering osmotic pressure from the ideal by effectively reducing particle number. For solutions of macromolecules (e.g. protein), solute-solvent interaction is predominant, raising osmotic pressure by effectively raising concentration of solute as macromolecular micelles trap solvent.

types of attraction or interaction: solute-solvent interaction in which the solute attracts other solute molecules. When solute-solute interaction predominates less water interacts with or is bound to the solute resulting in a lower osmotic pressure since the water is more free to transfer across the membrane. When solute-solvent interaction predominates and is greater than one would expect from the concentration of the solute, the osmotic pressure will be higher. One should remember that the factor responsible for dissolving a solute in a solvent is solute-solvent interaction, which for an "ideal" solution results in a linear relationship between the particle concentration and the osmotic pressure.

Macromolecules, which cannot pass through cell membranes, hold water in the body. Macromolecules manifest increased solute-solvent interaction, resulting in very high osmotic pressure. The osmotic pressure-concentration curve for the protein macromolecule albumin which is responsible for maintaining plasma volume is shown (Fig. 3). The curve for albumin is a simulation of previous data (Scatchard *et al.*, 1946).

The oncotic pressure of blood plasma with 5 gm/dl albumin is about 25 mm Hg. A five-fold increase in concentration to 25 gm/dl will result in more than a 25-fold increase in osmotic pressure to over 600 mm Hg (almost one atmosphere). Hemoglobin is the major macromolecule in the erythrocyte. It has a molecular weight nearly equivalent to that of albumin, about 68,000. This, it might be presumed that the oncotic pressure of hemoglobin would be about equal to that of albumin. The concentration of hemoglobin within the erythrocyte is about 34 gm/dl; this should produce an oncotic pressure of several atmospheres, according to the oncotic pressure concentration curve. Obviously, cell membranes cannot withstand pressures of that magnitude without bursting. What mechanism reduces the intracellular pressure to more reasonable levels? The usual explanation is that the cell membrane has active transport capability and can pump small micromolecules out of the cell to lower the osmotic pressure within the cell. Although this may be a factor, we have postulated that the oncotic pressure within the cell may be considerably less than expected because of increased solute-solute interaction at the high intracellular protein concentration.

The high concentration of solute molecules would result in greater solute-solute interaction and a lowered osmotic pressure. In fact, one could extend that concept to the point

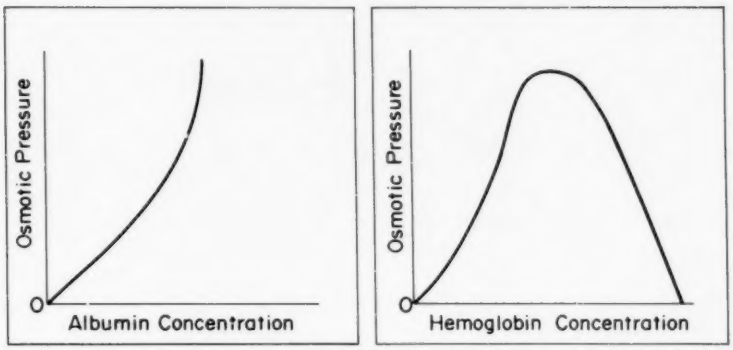

FIG. 3. (Left) Osmotic pressure curve of extracellular albumin might suggest that high concentrations of proteins within the cell would generate intolerably high pressures incompatible with cell viability; (Right) Increased solute-solute interaction at very high solute concentrations may be responsible for less-than-expected intracellular osmotic pressure curve of hemoglobin.

where osmotic pressure would begin to decrease as solute concentration increased to very high levels, i.e. when the solute was actually aggregating. The concept could be extended further to the point where osmotic pressure would approach zero, meaning that the solute was no longer in solution, but precipitated out. One could even visualize a system in which the osmotic pressure would become negative, signifying that solvent was being hydrophobically squeezed out of a precipitating or crystallizing solute; as a result the solvent might then be in a metastable state, in which it would be transiently more reactive chemically than pure solvent. Water of crystallization may be the end result of such a process in which the water has been induced to combine in a fixed proportion at certain sites on the crystallized solute. In fact, pilot experiments by the author have shown that the osmotic pressure at high hemoglobin concentration (25 gm/dl) does tend to be lower than expected. The finding is in accord with a recent report (Prouty *et al.*, 1985) revealing a change in the slope of the oncotic pressure/concentration curve for hemoglobin S at high concentrations. These experiments have not yet been extended past 30 gm/dl to approach normal intracellular concentration because of technical difficulties in working with highly viscous solutions.

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However, a pathophysiologic model exists in sickle cell anemia, which we believe demonstrates the phenomenon of decreased osmotic pressure resulting from solute-solute interaction. When exposed to a low partial pressure of oxygen, the hemoglobin S molecules tend to stick together forming a giant aggregate called a tactoid. The sequence of events in the sickling phenomenon is illustrated (Fig. 4). The sickling cell initially shows the tactoid with a clear space between the tactoid and the cell membrane. As the process continues, the clear space disappears and the membrane collapses around the tactoid. The clear space appears to be cell water, which diffuses out of the cell since the solute-solute interaction of the aggregating hemoglobin—taking the hemoglobin out of solution—reduces the osmotic pressure within the cell to the point where solvent (water) cannot be retained within the sickled cell.

We postulate that in aging the macromolecules within cells undergo increased solute-solute interaction with a consequent lowering of intracellular oncotic pressure. This could

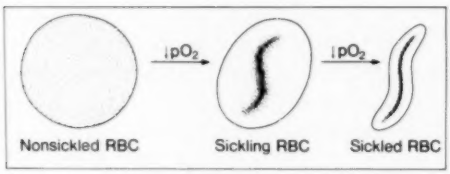

FIG. 4. Sickle cell anemia provides model for hypothetical role of osmotic water loss in aging cells. High level of solute-solute interaction of hemoglobin molecules leads, at low oxygen tensions, to aggregation of hemoglobin molecules into a tactoid surrounded by cell water. As aggregation reduces osmotic pressure, water diffuses out of cell and cell membrane collapses around the hemoglobin tactoid.

result in water loss from the cell causing a higher concentration of macromolecules which would result in even greater solute-solute interaction and further lowering of oncotic pressure with even greater water loss. If ways could be found to reduce this interaction the cycle might be interrupted.

Obviously, what is needed is a careful investigation of the chemical potential of tissue cell water as a function of age.

EXPERIMENTAL APPROACH

Fresh bovine ocular lenses obtained by extracting the lenses from globes removed from calves (less than two weeks old) and adults (greater than two years old) which have been freshly slaughtered for human consumption, are being utilized. Early results reveal that colloid osmotic pressures of lenses that had not been extracted from the globes for several hours after slaughter and globe removal, were somewhat lower depending upon time of removal, presumably because of imbibition of water from the anterior chamber aqueous humor and/or from the vitreous, as the epithelial "pump" became less efficient with time following demise of the animal.

Following extraction of the counted lenses, the capsules are removed and the lenses placed in a preweighed polyethylene container and conglomerately weighed in their closed collection container. The collection container is subjected to vortex mixing by a Scientific Products S8220 Deluxe Mixer. This separates the cortices which form a viscous solution in which the dense lens nuclei discretely remain. The discrete nuclei are then plucked out with a small covering of cortex solution around them, and placed in a separate preweighed polyethylene container and conglomerately weighed in their closed collection container.

Aliquots of homogenized cortex are placed in small closed sample tubes and frozen until colloid osmotic pressures can be measured.

The lens nuclei are then macerated by hand. Aliquots of the extremely viscous nuclei homogenate are placed in small closed sample tubes and frozen until colloid osmotic pressures can be measured.

Colloid osmotic pressures are measured in a rapid automatic semi-micro membrane colloid osmometer* utilizing Schleicher and Schuell Cellulose Acetate AC 62 membranes with 20,000 molecular weight retentivity via 0.010–0.005 μm pore size.

*U.S. Patent #4475556, Oct. 9, 1984, Theodore R. Reiff "Rapid Measurement of Colloid Osmotic Equilibrium Pressure." U.S. Patent #3063288, November 1962, Theodore R. Reiff "An Automatic Semi-Micro Colloid Osmometer."

At 1 mm diameter flexible polyvinyl fiber stirrer driven by an electric motor is centrally inserted through the osmometer solution chamber cap, positioned just above the membrane to prevent unstirred layer effects which are noted to be quite prominent without use of the stirrer. Osmotic equilibrium is reached within 1–2 h on virtually all specimens. Measurements are made in a water bath kept at 24°C.

The unstirred layer effects are quite large resulting in lack of attainment of equilibrium because of slow solvent diffusion through the viscous solution. After equilibrium is reached with stirring, if the stirring is discontinued there is about a 10% reduction in measured equilibrium pressure presumably due to concentration of solute at the upper portion of the solution chamber with concentration of solvent at the bottom of the solution chamber just above the semipermeable membrane.

RESULTS AND DISCUSSION

Preliminary results on noncataractous lenses reveal, as expected, much larger colloid osmotic pressure of the lenticular nuclear homogenate as compared with the cortical homogenate. This is consistent with the known increased protein concentration in going form cortex to nucleus. The colloid osmotic pressure data for noncataractous bovine lenses reveals values in the range of 2.5–3.0 $\times$ 10^5 Dynes/cm^2 for lens cortices and 5.0–5.5 $\times$ 10^5 Dynes/cm^2 for lens nuclei that do contain a small amount of adherent cortical material, i.e. the lens nuclei have about twice the colloid osmotic pressure of cortical homogenate. According to our hypothesis the colloid osmotic pressure of cataractous nuclei should be lower than that of non cataractous nuclei.

Attempts to measure the equilibrium colloid osmotic pressures of cataractous nuclei have not yet been performed because the homogenized nuclear cataract has been too dense and pastelike to permit mixing by the stirrer. Without mixing, the unstirred layer effect prevents attainment of equilibrium pressure within reasonable times. Our plan is to dilute the cataractous nuclear homogenate to a dilution that allows stirring and compare colloid osmotic pressures with equally diluted noncataractous nuclear homogenate.

Recent observations (Bettelheim *et al.*, 1986) that nonfreezable water content of cataractous lenses is less than in noncataractous lenses, are consistent with our hypothesis of decreased colloid osmotic pressure due to macromolecular aggregation. The extruded water Bettelheim refers to as freezable water, appears to be due to syneresis and manifests as clefts in the cataractous lens.

Physiological consequences of body water loss

The physiological consequences of any loss of body water would indeed be profound. If one considers some of the functions of water in the body, it will become apparent that the loss of water with age can effect many different functions.

One of the prime functions of water is to act as a diluent for water soluble medication. Since the therapeutic effect and the toxicity of medication is related to concentration within body fluids, it will become evident that if the same dosage of water soluble drug is given to an older individual as to a younger person the drug will be distributed in a smaller volume of distribution in the older person resulting in a higher concentration with greater therapeutic and greater toxic potential. This can be demonstrated simply by the equation concentration equals mass of drug administered/volume of distribution. This implies that older persons may need lower dosages of water soluble drugs to achieve the same therapeutic efforts as would be required in younger persons. In addition, other influences

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tend to make older persons more susceptible to drug toxicity since they may excrete water soluble drugs more slowly because of decreases in renal function with age and because they may metabolize drugs more slowly due to decreases in hepatic function and in peripheral tissue degradation.

Another function of water in the body is to act as a thermal buffer. Water is well adapted to this function because of its high specific heat, i.e. the number of calories required to be removed or added to effect a change in temperature of one degree per gram. Water has a specific heat of one calorie per gram degree centigrade. Metals have much lower specific heats. If one takes a hundred grams of iron and places it in a glass and a hundred grams of water and places it in another glass and puts both of the glasses under the windshield of an automobile in the summer the glasses will each receive the same amount of radiant heat from the sun. If one returns in an hour and touches the metal, a burn may ensue. If one puts their finger in the water, the water will be warm but not hot enough to inflict a burn and, in fact, could even cool the burn received by touching the metal. The reason for this is, although both glasses receive the same amount of radiant energy, the temperature of the iron rose much more than that of the water because its specific heat was less. By the same token if one was to place an eighty year old and a thirty year old out in the sun and come back in several hours one might find that the older person has developed environmental hyperthermia. A reason for this is that the older person has less water and, therefore, less thermal buffering capacity and body temperature rises more than that of the younger person who has much more body water to absorb some of the radiant energy without raising the temperature of the individual. Alternatively if one were to expose the eighty year old and the thirty year old to winter temperature, the older individual would develop environmental hypothermia much more rapidly than the younger person since the older person doesn't have a reservoir of body heat contained in a large amount of body water as the younger person does. This means that older persons are more susceptible to environmental hypothermia and environmental hyperthermia than are younger persons. Of course, there are other reasons for the increase in sensitivity of older persons to hyper and hypothermia, related to central nervous system control, decreased sweating, etc.

A decrease in total body water of older persons makes them more susceptible to dehydration since they have less of a reserve of water to fall back on. In addition, older persons, because they have less capacity to store body water, are more susceptible to over hydration than are younger persons because they have a smaller volume of distribution in which to dilute exogenously administered water.

Recently it has been shown (Phillips *et al.*, 1984) that healthy elderly men have reduced thirst after water deprivation as compared to thirst control in younger persons. Twenty-four hours of water deprivation was carried out, studying plasma osmolality and plasma vasopressin levels along with urine osmolality and sensations of thirst and mouth dryness. There was clear evidence that older persons, although exhibiting slightly higher serum osmolality and vasopressin levels, did not express thirst or mouth dryness as did the younger persons. This would indicate that older persons would have a tendency to ingest, and actually they did ingest, less water in response to water deprivation than did younger persons. This meaningful study further demonstrates that older persons are at considerably more risk for dehydration not only due to their decreased body water content, but also due to the fact that they seem to experience reduced thirst sensations. It had pre-

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viously been shown (Helderman *et al.*, 1978) that older persons responded less well to vasopressin and this was felt to be due to decreased renal response, presumably because of decreased renal function with age. The known hypertrophy of the supraoptic and paraventricular hypothalamic nuclei, the sites of control of vasopressin release, could be secondary to senescent tissue water loss.

Very often these physiologic differences between elderly and younger patients are insufficiently recognized and attended to, resulting in deleterious effects. No one would think of giving infants the same dose of medication as given to adults, but relatively few physicians take into adequate consideration the physiological differences of body water and other body compositions changes when prescribing for the elderly.

Another effect of the decrease in body water on age is that older persons become much more sensitive to and susceptible to hypovolemia and dehydration related to diuretic therapy and to dialysis. With the increased emphasis on treatment of hypertension in the elderly, including the treatment of isolated systolic hypertension, there is a tendency to over prescribe powerful diuretic therapy for older persons. While younger persons may tolerate daily diuretic medication, very often the systolic hypertension in the elderly can be controlled with every other day or every third day diuretic therapy. It is incumbent upon the physician to adjust medication assiduously and to monitor the older patient more carefully than is needed with younger patients.

If the colloid osmotic hypothesis of water loss in aging is substantiated an important corollary is that dehydration would increase the degree of solute-solute interaction with macromolecular aggregation, a certain percentage of which might be irreversible even with rehydration. This could lead to permanent tissue damage. Methods to reduce macromolecular solute-solute interaction and to maintain tissue water would become important. Whether or not the hypothesis is substantiated, it is clear that dehydration is more hazardous in old than in young individuals. Great care should be taken to avoid dehydration in senescent individuals.

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AGE-RELATED POLYPLOIDIZATION OF HEPATOCYTES: THE CAUSE AND POSSIBLE ROLE

A Mini-Review

ZHORES A. MEDVEDEV

Genetics Division, National Institute for Medical Research, Mill Hill, London NW7 1AA, Great Britain

Key Words: aging, polyploidization, liver, hepatocytes, detoxification

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INTRODUCTION

THE RAT and mouse livers represent the most widely used tissues to study the age changes of general metabolism, enzyme activity, age-related changes of DNA, RNA and protein synthesis, and many other physiological, biochemical and functional characteristics.

Livers of birds, reptilia, most amphibia and fish species normally consist of diploid parenchymal cells (hepatocytes) which make up to 80–90% of the liver biomass. Other cell types which are essential for liver function (endothelial, Kupffer and fibroblasts) are also diploid. The hepatocytes of mammalian species however, show an early and intensive polyploidization (formation of binuclear, tetraploid, tetrapolid binuclear, octaploid and higher ploidy classes) which has been clearly shown to be an age-related process.

In 1958–1959 when Nathan Shock included rat liver in an already substantial list of organs and functions being tested for age-related changes in the laboratories of the Gerontology Research Center at the Baltimore City Hospitals, it was not then clear whether the age changes of liver functions were a direct and primary result of the senescence of hepatocytes or if they could be partly related to their polyploidization. The pattern of polyploidization was not identical in different species. It was found that the occurrence of progressive polyploidization in human liver is restricted to the periods of active growth and development and does not continue beyond the adulthood (Swartz, 1956), while in mouse liver the accumulation of hepatocytes with increasing ploidy levels continued during senescence as well (Andrew *et al.*, 1943; Andrew, 1952). It has been shown that there is a rapid increase in the proportion of tetraploid and octaploid hepatocytes in rat liver during the postnatal period, but the pattern of hepatocyte polyploidization in adults and during senescence was not studied. Shock and his collaborators (Falzone *et al.*, 1958; 1959) were the first to show that the pattern of polyploidization of hepatocytes in rat liver was similar to that obtained from human liver. It was progressive only until 12–14 months of age and did not continue

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into senescence. There were no further changes in the average hepatocyte nuclear volume and the nuclear DNA content, between 14 and 27 months of age. The average nuclear volume and DNA content correspond to that of tetraploid class. These findings suggest a possible link of changes of liver functions after 12–14 months of age with senescence and not with progressive polyploidization. Later studies (Carriere, 1969; Van Bezooijen *et al.*, 1972/73) confirmed these results. It was also confirmed that the polyploidization of parenchymal liver cells is more extensive in mouse livers and does not stop or become reduced during adulthood. The senescence period (age between 14 and 28 months) coincides with the accumulation of 8C, 16C and 32C nuclei (Shima and Sugahara, 1976; Brodsky and Uryvaeva, 1977). Even during the later stages of senescence (28–33 months of age in CBA mice strain) when the liver biomass starts to decline there is a continuing accumulation of highly polyploid 32C and 64C hepatocyte nuclei (Medvedev and Medvedeva, 1985).

Age-related polyploidization and binuclearity was observed in some other mammalian tissues, particularly those with secretory functions (pancreatic, thyroid, pituitary, submaxillary and parotid glands) (Brodsky, 1964). However, it is only in mammalian liver where polyploidization starts almost immediately after birth and continues in some species throughout their lifespan (Gahan and Middleton, 1982). It is therefore, important for studies on liver aging to answer some basic questions. The first of them naturally, is about the physiological or functional role of progressive hepatocyte polyploidization in mammalian species. The second is about the relevance of the polyploidization for the general process of senescence. One may find it very surprising that although the mammalian liver is the most favoured organ for gerontological studies, the answers to both questions are still hypothetical.

THE ROLE OF THE AGE-RELATED POLYPLOIDIZATION OF HEPATOCYTES

Diploid hepatocytes which are functionally active are also liver stem cells producing new hepatocytes during growth and regeneration. Because hepatocytes absorb some toxic products as part of their function, it is possible that liver cells accumulate genetic damage more rapidly than cells of many other tissues. The absence of a special pool of nondifferentiated stem cells able to produce new hepatocytes makes the polyploidization probably a safer way of replacement of cellular losses than mitosis which may result in a high incidence of chromosomal abnormalities. Brodsky (1964) considered somatic polyploidization in liver and some other tissues as a normal form of so called "physiological regeneration". This view corresponds well with the negative correlation between the mitotic index in liver and the level of polyploidization found in different species (MacDonald, 1961; Carriere, 1969). Mouse liver has a much lower mitotic index than rat liver and accordingly the highest level of hepatocyte polyploidiation was found in mouse liver. Polyploidization is minimal in guinea pig livers which also have the highest mitotic index of hepatocytes amongst rodents. Polyploidization, being a kind of regeneration, makes it possible to preserve the normal size of the liver and the approximate volume of its functional capability without a risk of accumulation of a large proportion of aberrant, aneuploid cells, which may increase the incidence of cellular transformation. However, the fact that polyploidization in livers of most mammalian species starts very early, often after birth and during the period of active growth suggests a protective role of polyploidization (reducing the occurrence of mitoses which represent the most sensitive stage of the cell cycle and decreasing the cellular accumulation of aberrant genomes, rather than providing an alternative way of repair and re-

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generation). Several hypotheses have been proposed to explain the possible mechanism of the protective role of polyploidization, either by the creation of redundant genetic information and reiteration of vital genes (Medvedev, 1972; 1973), or making the cell more resistant to toxins and carcinogens (Evans, 1976; Benedict and Johnes, 1979); or as a morphogenetic strategy against rapid aging in specialized diploid systems (Gahan, 1977). Polyploidization has been also considered as a cellular process reducing deleterious effects of chromosomal aberrations which might have higher frequency in liver resulting from their detoxification functions (Uryvaeva, 1981). The protective role of the polyploidization is consistent with the observations of the higher stability of polyploid plant and animal cells *in vivo* and *in vitro* to the effect of radiation and chemical mutagens (Sparrow, 1962; Simon, 1965; Chasin, 1973; McBurney and Whitmore, 1974; Bauer, 1974). Some authors suggest that in addition to the protective role of polyploidization, it also makes cells more efficient due to the increase in the nuclear/cytoplasmic ratio (Tauchi and Sato, 1962, 1962; Brodsky, 1964; Gahan and Middleton, 1982). However, it is unlikely that the "productivity" of one octaploid cell is greater than four diploid cells. Because the surface area/volume ratio of polyploid nuclei is less than that for diploid cells (in 8C nuclei the surface is increasing only by factor 2.5), the nuclear/cytoplasmic exchange per unit of nuclear mass should show a reverse correlation with the size of nucleus. Fewer numbers of larger cells in old livers, are hardly expected to perform better than more numerous but smaller cells in young livers. For example, it is known that the liver "dye storage capacity" which is considered as a physiological indicator of liver function, declines with age. It was found that this decline correlates with the progression of hepatocyte polyploidization (Nakanishi *et al.*, 1980). The age-related decline in the liver efficiency of detoxification is, at least partically, connected with polyploidization. The early postnatal polyploidization of hepatocytes may have a protective role which increases the life span of individual hepatocytes. However, the polyploidization of hepatocytes in later periods of life, particularly the progressive polyploidization which is observed in mouse liver during senescence by formation of 8C, 16C, 32C nuclei, is apparently an integral part of the aging process. As such, it may not represent a real protection against aging, but a tissue-specific adaptation to the age-related cellular loss. If this suggestion is correct, it may be that the acceleration of hepatocyte polyploidization would occur not only during senescence of mouse and some other rodents, but in the presence of dietary hepatotoxic substances. This expectation has been confirmed in our recent study of the effects of a toxic and carcinogenic diet on the pattern of hepatocyte polyploid ization in mouse liver (Medvedev *et al.*, 1986). It may be feasible to use the pattern of polyploidization in mouse liver as a sensitive test of the hepatotixicity of different drugs and chemicals.

There is some data indicating that hepatocytes are subjected to a higher level of genetic damage than cells in other tissues. The accumulation of single strand breaks in DNA is higher in hepatocytes than in other tissues (Ono and Okada, 1979). Mitotic division, induced during regeneration, also shows a much higher level of chromosomal aberration in liver than in other tissues during their proliferation. In young mice about 20% of mitosis in regenerating liver show chromosomal aberrations and from 40 to 80% in regenerating livers of old animals (Crowley and Curtis, 1963; Curtis, 1966; Brooks *et al.*, 1973).

But if hepatocyte polyploidy in mammals plays a protective role against the toxic, deleterious and mutagenic effects of chemicals and metabolites, and provides organisms with a safe alternative to replacement of age-related loss of cells, why has hepatocyte polyploidization appeared so late in evolution and only in the mammalian species. How are birds,

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reptilia, amphibia, fish and many other vertebrate species, often with very long life spans, able to protect their liver function without polyploidization of hepatocytes?

AGE-RELATED POLYPLOIDIZATION OF HEPATOCYTES AND THE EVOLUTION OF THE DETOXIFICATION IN VERTEBRATES

Livers in different species are able to detoxify very many different natural and synthetic toxic substances, but the evolution of the "detoxification metabolic system" apparently occurred first in response to the toxic products which are always present in cells and in blood circulation and which are generated by the normal functions of living cells. Safe elimination of toxic end products of *normal* metabolism is a priority and CO_2 (the energy cycle) and NH_3 (the protein and aminoacid metabolism) are the most essential. If these two simple molecules which are formed in all living cells are not constantly removed, cells die in a matter of minutes. For all aquatic vertebrate species, the removal of CO_2 and ammonia is comparatively simple and not related to the detoxification reactions. They are removed by diffusion into the surrounding water and the process is accelerated by the special gill functions (external, lamellar, pectinate and some others). However amphibian species have a more complex detoxification for ammonia. Tadpoles excrete ammonia into water, but frogs convert ammonia into urea which is less toxic and which can be partly diffused into water and partly excreted. Liver is the organ where this synthesis of urea takes place and seasonal polyploidization of hepatocytes up to tetraploid level occurs in some amphibian species, particularly in hylid frogs and only during the breeding season (Bachmann *et al.*, 1966). However, the formation of urea is replaced by the formation of uric acid in reptilian species and birds, most probably because uric acid is less soluble and less toxic than urea. [Some authors consider uric acid to be an antioxidant which enhances the lifespan opportunities in these species (Cutler, 1982)]. The detoxification of ammonia by the synthesis of uric acid apparently does not reach a toxic level in liver which may damage diploid hepatocytes. However, the very low solubility of uric acid makes it necessary to remove this product by the cloacal type of excretion common in birds and reptilians. It is interesting to mention that the ammonia concentration normally increases in the early stages of the chick embryo, but is then replaced by a phase of urea formation, which in turn is substituted by a final "adult" uric acid-producing detoxification mechanism (Meister, 1965). This may explain why the binuclearity and polyploidy of hepatocytes appears in the livers of chicken embryos, but towards the end of embryonic development the proportion of tetraploid and octaploid hepatocytes declines (Medda *et al.*, 1984). This unusual "depolyploidization" during the later stages of embryonic development of the chick could be directly related to the switching off of the urea formation and switching on of less toxic uric acid method of ammonia detoxification. A return to the urea cycle for detoxification of ammonia by mammals had no specific biochemical advantages, being most probably linked with the particular "life styles" of mammalian species. These made it necessary to accumulate in their bodies comparatively large amounts of excretory products which could only be disposed of into the environment at random intervals. The less frequent disposal of excretion products was solved in evolution by the formation of a separate urinary system for which the uric acid, because of its low solubility, was not a suitable product. There was no other alternative, but the re-emergence of urea, which is less toxic than ammonia, but more toxic than uric acid. The detoxification of ammonia by conversion to other excretion products was a necessary adaptation during evolution from an aquatic existence to life on land. It could

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also be accomplished by other biochemical conversions, for example, plants normally store amino acid amids as a safe reserve of nitrogen from ammonia and do not excrete them, in spiders a guanine is the major nitrogen excretion product. It is not our task to explain why urea was "chosen" by the evolution for mammalian species despite some toxic disadvantages. Ammonia which is continuously released from cells into circulation is produced by the deamidation of amino acids, purines and pyrimidines, of asparagine and glutamine and even of proteins (Meister, 1965). Port blood normally contains a much higher concentration of ammonia than the peripheral blood. Transformation of ammonia into urea in liver certainly makes liver cells more exposed to the toxic effect of ammonia and urea and the urea cycle of detoxification is the most essential among many different liver functions. Neither ammonia nor urea, however are mutagens. It is plausible to consider the higher toxicity of the urea cycle in comparison with the uric acid cycle as the evolutionary justification of early polyploidization of hepatocytes. This means that the hepatocyte polyploidization is a special type of self-protection (and later a consequence as well) from the toxic effect of ammonia and urea, not a defence against the mutagenic products of the environment. The theory of autointoxification as a cause of aging (Metchnikoff, 1907) considered some toxic products of bacterial origin in the large intestine as the main cause of aging. It is possible to argue, however, that the same rate of aging occurs in rodents kept normally in bacteria-free environment (germ-free animals), indicating that the bacterial sources of toxic damage are probably too insignificant in comparison with toxic products of normal metabolism. (It could be interesting to compare the level of hepatocyte polyploidization in germ-free and normal mice). However, modern industrialized societies are linked with the appearance in the environment and in food of very many toxic and carcinogenic products. Many of these compounds are detoxified in the liver with different efficiencies. Quite a few of them cannot be destroyed by the existing detoxification enzymatic systems in liver. Some products could be more toxic for species with deploid hepatocytes, but less toxic for polyploid hepatocytes usual in mammalian species. In many cases therefore, young mammals may be less tolerant to environmental toxic substances than adults in which polyploid hepatocytes are more resistant. Older animals are again more sensitive to different pollutants and drugs, but probably not because their highly polyploid cells offer less protection, but because they are simply less efficient metabolically.

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GENETIC INSTABILITY AS THE PRIMARY CAUSE OF HUMAN AGING

BERNARD L. STREHLER

Molecular Biology Division, Department of Biology, University Southern California, Los Angeles, California, 90089

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Abstract—The origins of aging of higher forms of life, particularly humans, is presented as the consequence of an evolved balance between 4 specific kinds of dysfunction-producing events and 4 kinds of evolved counteracting effects in long-lived forms. Among the deleterious events, particular importance is assigned to damage to DNA, but damage of a different kind than classical mutations. The evolution in man's ancestors of means to counteract the kinds of events that limit the life-spans of short lived mammals is postulated to be the indirect consequence of the prior evolution of superior mental capacities. Further, it is shown that the human species rapidly evolved its life-extending mutations because of the special circumstances afforded by the subdivision of the species into small semi-isolated (genetically) tribes of 10–100 individuals in which polygamy was the key factor in rapid incorporation of life- and well-being-extending new features. These conditions permit at least one or two orders of magnitude more of such beneficial genes to have been incorporated into our genomes during the 100,000 years or so of extremely rapid human evolution that evidently occurred about 100,000 to 200,000 years age than has been posited by other workers. The sources of damage to DNA are then considered, with special emphasis on free-radical derivatives of molecular oxygen and evidence is presented that longer lived forms of mammals have peroxide lysing enzymes that produce a lower steady state of damaging radicals derived from this compound. Evidence that so-called "classical" mutations cannot be the source of aging is then reviewed. A different kind of mutation, one that is not increased in proportion to point mutations by mutagens, namely deletion of tandemly duplicated copies of genes, is discussed and the evidence that such damage (gene loss) occurs in an amount sufficient to account for the major losses in function during aging is presented. The most likely mechanisms of such loss plus the prospects for evolving and bio-engineering means to counteract these losses together with some implications regarding the documented loss of of NORs with age (as regards rDNA loss) together with key areas for intensive present and future research on aging are presented.

"The proper way to create a statue is to remove the unneeded substance"
—paraphrased after Michaelangelo

INTRODUCTION

ANY SYSTEM that is not in thermodynamic equilibrium will approach that state at a rate that is a function of absolute temperature and the energy barriers to the rearrangements of

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components. Because living beings are not in such a state of equilibrium, they gradually change in their internal structures as time passes—unless they are able to use the energy (and materials) available to them to restore structures as they change. Only a few forms of life possess the ability, as individual entities, to replace all of their constitutent parts and thus to live indefinitely long without evidence of deterioration.

Any organized structure that is not in thermodynamic equilibrium will also gradually become disorganized—unless potential energy is lost when order is generated. Crystal formation is one example of such an organizing process and differs from the organization that living things are able to generate in that the specific dissolved components that can give rise to a crystal have affinities for each other—which affinities are sufficient to overcome the randomizing-disordering effects of ambient kinetic energy (heat). Because no particular arrangement is more probable than any other, a gradual loss of order will occur, essentially because there is almost an infinity of disordered arrangements in which a system can exist and only one way in which any particular kind of ordered state can exist.

The answer to the puzzle of aging among living things derives from the above general laws of physics, but the laws, of course, do not specify which kind of change, if any, dominates the aging process. In fact, any conceivable disruption will take place at some rate, great or small, so that in a sense any imaginable theory of aging possesses at least some tiny grain of truth.

ON THE EVOLUTION OF LONGEVITY

The basic question that guides critical research on the origins of aging is then not whether a given deteriorative process is part of aging, but rather whether it is a sufficiently large and rapid process to have a significant effect on the function of a biosystem that shows deterioration as time passes. In order to approach a puzzle that has so many facets and possible origins it has been found economical of time and resources first to define some key parameters of the aging system under study. These parameters typically include measurements of the viability of the system at different ages; viability is usually measured by determining the death rate at different ages. If the death rate is constant, a first order exponential decline of survivors is observed vs. time. Such a constant death rate does not necessarily mean that such systems are not undergoing aging, but only that they do not age rapidly enough in that environment for age changes to affect mortality rates. Many animals in the wild show such constant death rates essentially because they are subject to predation or other destructive forces to such an extent that they never have a "chance" to age (Comfort, 1956; Flower, 1931).

Many simpler animals, including some vertebrates, produce large numbers of offspring, most of which are "expendable", their places in the reproductive pool being taken by siblings or other relatives that escaped destruction before reproductive age was reached. But, because an advantage is conferred, in terms of reproductive perpetuation, on any variants that manage to escape destruction because of larger size or better defense than potential predators, one very successful line of evolution has taken the form of animals of greater size.

The evolutionary risk implicit in the increase in size of members of a species is that reduced numbers of offspring must be produced, for a given niche can only accommodate some approximate maximum total mass of a "niche-occupying" species. If a large number of large animals attempts to occupy the same space as was previously "optimally" occupied by the

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same number of similar but smaller animals, the large species will be at the edge of starvation and may occasionally even become extinct because it may destroy its own food supply. Additionally, (except for advantages it has against competing species or predators because of size) a large species comprising fewer total individuals than its smaller version is at greater risk of extinction than is a small form of the same animal. A few members of the small species will have a better chance to survive a crisis in food supply or the inroads of a new and more efficient predator or disease.

In addition to increase in size and the development of offensive or defensive means (the latter for example, protective shells in tortoises) there was an advantage (particularly great among predatory species) to the storage of past experience in recallable memory. The memory of which hunting strategy was successful (and which was not) was vastly superior to random choices among different food-seeking or danger-avoiding strategies. This permitted many predatory species to survive even in times of low average food supplies. This advantage of access to stored memories led to relatively rapid increases in brain size and intelligence—the ability to manipulate and compare memories of the immediate past* with those representing the more distant past.

As the ability to benefit from past individual experience expanded, the death rate from starvation and from predators decreased—so that such species began to include some members that lived long enough to begin to show signs of aging. It is here suggested that it only became of benefit to evolve an increase in species longevity after the brain had evolved to a sufficient degree that sufficient numbers of individuals lived long enough to approach the species limit on longevity. Sacher (1975) has carefully documented the relationship between the brain/body weight ratio and species longevity among mammals. This relationship is not evident among reptiles such as tortoises, presumably because they are so well protected against predators by their shells that there is little advantage to being quick-witted or intelligent. On the other hand, among larger and agile birds, such as owls and parrots, the ability to remember multiple specific locations in which food may be found and where congenial or safe living conditions exist provided a similar impetus to an expanded memory capacity—and also to smaller numbers of offspring, accompanied by the greatest longevities recorded among birds.

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The evolution of man from very distantly related tree-dwelling primates probably owes much to the fact that there is a vital advantage to being able to store in memory the locations of myriads of branches with respect to each other—a feature that permitted the outwitting of carnivorous climbing predators. Even after man's predecessors moved to grassy plains or sea-side habitats, the previously evolved memory system served these more immediate ancestors well, as did the evolution of an upright stance. Although some carnivores and even pachyderms spend much time educating their young, the long term care and education of tree- and ground-dwelling primates was an absolute necessity for species survival. The social groups that fostered extensive rearing of the young also probably led to the invention/evolution of symbolic communication through speech among our more immediate ancestors, particularly when a communal/tribal life mode was adopted. The invention of spoken language made it possible for an animal to learn quite a bit about the world with-

*Note: In a sense there is no real, present time. What we perceive as the present is what we have just experienced happening—plus what we predict will happen in the immediate future.

out ever experiencing some of its direst and most dangerous aspects. Speech also made an even larger brain, one suited to store and manipulate words and other symbols efficiently, necessary and of great benefit.

Each of these evolutionary improvements in brain function led to a species that had an ever increasing mastery over its environment and to the security against destruction that accompanies such mastery. This, in turn, placed (perhaps for the first time) a substantial advantage on the evolution of greater and greater longevity so as, as it were, to optimize the usefulness of mental capacity in the reproductive propagation of the evolving species.

There seems little doubt that our closest relatives among the primates have substantially greater intrinsic lifespans than our more distant primate relatives had. And, according to Cutler's imaginative analysis (Cutler, 1976), there is also little doubt that there was an almost explosive increase in longevity among our most immediate ancestors about 100,000–200,000 years ago. There is, of course, little if any advantage for a species to evolve a body that can live for 100 years if it is so dull-witted that it dies of predation or improvidence in 5 years; conversely, there is little advantage to evolving the brain of a genius if the body supporting it deteriorates from aging before that mental capacity can bestow its benefits. Thus improved brain functions and increased longevity have evolved hand in hand. The road to increased longevity was, however, paved by the prior evolution of increased mental capacity—not vice versa.

In order for a shorter-lived species to evolve into a longer-lived one it is "only" necessary that new genes be evolved that will preserve the corpus for the use of the mind. The fact that the substantial increases in maximum human life span have occured during quite short evolutionary epochs has led some to the unnecessarily facile and simplistic conclusion that only a relatively few (10–200) mutations are responsible for the differences in longevity between man and the great apes. The logic used in such estimates takes the known mutation rates per gene per generation and calculates the number of such "beneficial" (i.e. longevity increasing) mutations that could have occured during the relatively short time in which these improvements occured. The conclusion that only a few such mutant genes led to this great increase in longevity has even led one worker in this field (Cutler, 1979) to state that the changes are not due to mutations in "structural" genes, but rather to modifications of so called "regulatory" genes (whatever these may ultimately turn out to be). The thesis behind this suggestion seems bizarre on the face of it, for at least the following two reasons: (1) The first is that in order for just a few such mutations to be the source of greatly increased longevity they must possess the unparalleled property that just a few of them were able to cause the generation of the multitudinous differences that exist between man and ape—e.g. brain size, subdivision of that structure into new separate regions, multiple new connections between areas of the brain, etc., physical size, bone shape and size, hair distribution or absence, muscle size and shape, menstruation, etc.; (2) even more devastating to this conjecture is that such genes must be selectively mutable.

The mutation number calculations assume an average gene mutation frequency. If only a few specific genes are involved then such hypothetical genes must have been specifically "chosen", through some imaginary or unknown mechanism. Stated otherwise, if only 10–250 mutated genes are responsible for our enhanced longevity, then these must have been selectively chosen out of the 20,000 to 100,000 genes available for mutation. Therefore the mutation frequency of these genes would have had to be 80 (20,000/250) to 10,000 (100,000/10) times greater than is observed for "ordinary" structural genes.

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The logical difficulty posed by the above assumptions and facts and the seemingly absurd requirements of that hypothesis can relatively simply and rationally be resolved if one takes into consideration the fact that mating is anything but random in an intelligent social species. The usual view of the mechanism through which beneficial gene "fixation" in a population takes place assumes that slight phenotypic advantages select for reproduction in direct proportion to the advantage conferred by that phenotype in areas of activity other than reproduction. This thesis is patently false because superior mental qualities not only can provide the incentives to mating between superior carriers of different and new beneficial genes (thus fixing these new genes very rapidly in such a self-selecting sub-population); selective-competitive mating can also give several orders of magnitude of reproductive advantage to a "beneficial" mutation carrying male who, though only sufficiently superior to his competitors to deny them procreative rights, may pass on his mutant heritage to tens or even hundreds of offspring through monopoly of the available females in the group. Computer simulation of the effects of such polygamy-monopoly shows that the time required for advantageous gene fixation may be reduced by a factor of 10 to 100 or more through the selective mating reproductive polygamy represents (See Fig. 1, after Strehler, 1979). The ratio of such reproductive advantage is essentially proportional to the polygamy ratio (females per "dominant" male.)

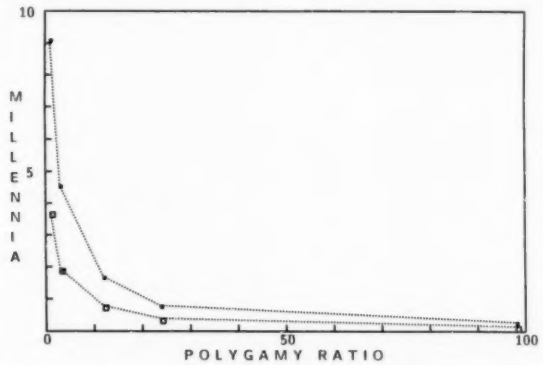

FIG. 1: The effects of polygamy on the time (in thousands of years) required for an advantageous gene to increase from 0.1% of the total number of alleles of that gene present to 90% of the total number are depicted. It will be seen that the effect of polygamy is to shorten the times required. The x axis defines the number of females/reproducing male in that society and the y axis the number of years (× 1000) for this increase to 90% to occur. In the case of monogamy (polygamy ratio = 1) it takes, for example, about 9000 years for a gene that confers an advantage of 1% on its male carriers to become preponderant in the population, but only about 3700 years if the phenotypic advantage is 2.5% as it affects ability to compete successfully up to the time of reproductive maturity. At higher polygamy ratios the time required is reduced roughly in proportion to the polygamy ratio. Thus, at a polygamy ratio of 25 it takes about 1000 years for gene fixation at a propagation advantage of 1% and about 300 years if the advantage is 2.5%. All of these calculations are based on a mean generation time of 10 years. The import of this graph (a computer simulation) is that nonrandom mating (in particular, polygamy) in a social species can very greatly reduce the time required for favorable traits to be incorporated into the genome of a small sub-group of a species (tribe). The implication is that the society in which our ancestors evolved particularly favored rapid evolution of human qualities (data taken from Strehler, 1979).

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Details: The mutation frequency per human generation is believed to be about 2–10 detectable (mostly harmful) new mutations in each new individual. If about 1% of such mutations are beneficial then about 5/100 = 1 in 20 individuals will possess one such gene. In a static population of 10,000 individuals there would therefore be 10,000/20 = 500 such new beneficial genes in each generation. If the average generation time were 10 years, then in 100,000 years 5,000,000 (10,000 × 500) such beneficial mutations would occur. Thus an advantageous mutation in every one of the 20,000 to 50,000 genes present in man's ancestors would have had an opportunity to occur about 100 times (5,000,000 mutations/50,000 genes) during such a 100,000 year period. Even if the total average population were only 1000 reproducing individuals, the number of times in 100,000 years that each gene could have mutated favorably would be 10 (per gene). If such a population is subdivided into 40 semi-genetically-isolated tribes consisting of 25 reproducing individuals each, then there would be one favorable and 100 deleterious mutations/tribe every 4 generations. Over the 30 generations (~300 years) required (at a polygamy ratio of 25) to fix such a mutation (so that 90% of the total alleles of the mutated gene would be of the advantageous variety in that time) the separate tribes would each acquire 1 such beneficial mutation as that one favorable mutation in the entire static poulation of 1000 individuals would be fixed in one of the tribes every 300/40 = 7.5 years. Therefore, because 100,000 years/7.5 (years for fixation of 1 favorable mutation in the total population) equals ca. 13,000, this number of favorable mutations could be incorporation into some part of the total population within the 100,000 years during which the highest rate of evolution of our species appears to have taken place.

In order for the favorable genes that arise in separate tribes to become part of the gene pool of the species as a whole, it is also necessary that the semi-genetic-isolation a tribe represents be broken occasionally so that different beneficial genes can become incorporated into a new, more highly evolved species. Polygamy will tend to make each tribe genetically homogeneous at the same time that it increases the rate of beneficial gene fixation. This occurs because if only a single male is the sire of all offspring in a generation in a tribe, than all of the individuals in the new generation will share the same father and share 50% of their genes in common. Because, in the next generation, the only possible matings are between siblings (or fathers and daughters), there will be rapid and effective selection against any harmful mutations provided by the grand-sire, while at the same time his advantageous qualities will also rapidly become predominant in the tribal line. Each tribe will therefore be relatively free of harmful recessives (because of strong selection against them) and each will tend to have acquired different sets of new beneficial ones as well.

It is important to keep in mind the fact that in a polygamous tribal society, the source of genetic diversity (new mutations, harmful or beneficial) is in the female members, because they represent a large proportion of individuals in the mating pool. Any new beneficial genes a newly "dominant" male acquires are almost certain to have arisen in his mother, not his father.

To return to the subject of wide dispersal of beneficial genes within the species, it is necessary that occasional intermixing and/or exchange of genes from each of the tribes take place. In earlier periods during this evolution, this probably involved raids on neighboring tribes for females and other benefits. It is ironic and romantically satisfying that forced intercourse with an invader simply enriches the diversity of the gene pool of the raided tribe, whereas the importing or seduction-abduction of a female from a neighboring tribe results in the introduction of any new beneficial genes she may carry into the gene pool of

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the tribe of which she then becomes a part. Such exchanges or abductions need not have been very frequent (e.g. once every 10 generations or so) in order to provide rapid gene intermingling within the species as it evolved. But, in order for maximum benefit to be achieved, each of the tribes must at least exchange or acquire genes with its neighboring ones at sufficiently close intervals to insure transfer of beneficial traits within the species as a whole. Those tribes at the "center" of such exchanges (i.e. whose neighbors have already incorporated beneficial genes from their other neighbors) will be at a particular advantage because those neighbors they have will already have accumulated and concentrated beneficial genes filtered through many inter-tribal matings.

In summary, firstly there is no valid reason to believe that the great number of specific phenotypic and behavioral differences between man and his closest relatives among the primates are the result of the incorporation of very few (10–250) new mutations. Secondly, there is no valid reason to believe that only a small number of life-span-extending mutations led to man's greatly increased maximum longevity over ancestral pre-humans. (This number may either be large or small, depending on the number of damage-counteracting advantageous mutations required to increase the time during which every essential body part maintains its functions.) The basic reasons for these conclusions are (1) that a social species that is divided into small, genetically isolated sub-groups (tribes) will not accumulate an excessive load of harmful recessive mutations (because in-breeding will tend rapidly to reduce the number of such mutations in each such sub-population); (2) that a high polygamy ratio greatly increases the effectiveness of the inbreeding needed to keep the accumulated burden of recessive harmful mutations low; (3) that polygamy simultaneously greatly increases the rate at which advantageous allelic mutations become dominant in such a tribal sub-population; and (4) that occasional outbreeding with members of a different tribal sub-population provides an efficient means through which the advantageous evolutionary "inventions" generated in each tribal sub-population can quickly become disseminated in the species population as a whole. Thus, sub-division of a social species into genetically semi-isolated, tribal, highly polygamous sub-populations (combined with occasional genetic exchanges between tribes) provides a means through which the rapid evolution of such a species can take place.

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Thus, arguments based on Haldane's mutation load limit concepts are not appropriate to such a special evolutionary setting because the total species population is sub-divided into small sub-populations in each of which deleterious mutations are rapidly eliminated through inbreeding while at the same time beneficial mutations can rapidly become the predominant alleles through the effects of polygamy. Also not relevant are simple arguments based on the number of accumulated amino-acid or genetic base substitutions (a few percent) separating man from his nearest living primate relative (the Chimpanzee). These are an inappropriate basis for making estimates of the number of key advantageous structural gene mutations that led to man's rapid evolution from prehominids because even a single base (0.1% change) substitution (e.g. sickling character in humans) can completely change the function of the product specified, as can single amino acid substitutions (ca. 1% change) in a protein specified by a structural gene. Other conservative factors such as the stability of codon usage over millions of generations (Strehler and North, 1981) will also tend to select against rapid gross changes in DNA sequences and against certain amino-acid substitutions. The central agent in man's rapid evolutionary ascent, then, was probably the genetic endowment that made a socially reasonably harmonious, highly polygamous, tribal life possible. The central effect of the environment created by

such tribal societies is the extremely rapid incorporation of desirable traits into the gene pool of the species as a whole as they occur.

These simple alternatives to the unrealistic assumption of random mating in pre-hominids thus provide a mean through which many thousands or tens of thousands of "beneficial" mutational differences could rapidly have become incorporated into the genomes of modern man's predecessors as the transition from pre-hominid to hominid to *Home sapiens* took place. The majority of such mutations no doubt provided the manual, lingual and pedal dexterities characteristic of man together with the superior mental and verbal and other symbolic communication capacities our species possesses, the latter based on a psyche attuned to the development of cooperative societies.

Such newly evolved qualities could not provide maximized benefits to early man if other modified or new genes whose roles were the generation of greater longevity had not also arisen. Some of this life extension probably occured through adjustments of the program of gene expression during the period between infancy and adulthood—the pre-adolescent pause in growth rate. Such a pause is beneficial to a social species because, inter alia, it causes an extended period of dependence of the young, a period during which the education of the immature animal for the assumption of an adult role can be instilled and during which social bonds and rules of behavior can be solidified. However, the extension of the childhood period adds only 5 to 10 years at most to the total lifespan and therefore accounts for only a small part of the increased maximum lifespan that differentiates man from all other primates. In order for the additional 50 years of longevity man possesses to evolve it was necessary for new genes to arise or for existing genes to be modified so as to provide this extended longevity. The roles of these new genes were to decrease the rate of failure of critical parts of the body. These new evolved qualities and the source of the age-generated failures that made their selection advantageous are discussed in the following paragraphs.

SOURCES OF FAILURE AND EVOLVED COMPENSATIONS

A systematic consideration of possible causes of failure of biological systems shows that they fall into just four major categories:

(1) *Gene repression:* The first of these is gene repression (the switching off) of genes whose products are needed to maintain function. If the products of such genes (or of many or most genes, in fact) each deteriorates at its own specific rate, then the system as a whole can only be maintained at a functional optimum if each deteriorating substance is supplemented or supplanted by new copies of that substance at a rate equal to its rate of deterioration.

(2) *Intracellular communication and spatial rearrangements:* The second source of decreased function derives from the spatial relationships between working parts of the system. Because there is an optimum spatial arrangement of the parts of a cell, tissue, organ system or body with respect to each other (an arrangement that provides for the rapid transfer of materials produced in one part of the society of cells and molecules that make up a functioning body to other parts of the body where they are utilized), any change in physical relationships or in barriers to diffusion, including circulatory ones, will result in some decrease in optimum function. Therefore slow "cell drift" within a highly organized tissue must be minimized through the firm anchoring of nonreplenishing cells to some supporting tissue skeleton and cells that are replaced regularly must come to occupy essentially the same positions as the original, replaced, cells had.

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(3) *Accretional defects:* The third contributing source of dysfunction during aging is the accumulation of waste materials composed of nonfunctioning or poorly functioning parts of the system. These substances may be intracellular, as is the case with the lipofuscin age pigments (Strehler *et al.*, 1959), may be extracellular as in the case of damaged collagen or amyloid accumulation or may be the debris represented by superfluous cells, as occurs during the thickening of the intima of blood vessels in certain age related circulatory diseases.

(4) *Depletional defects:* The final kind of age change that must be compensated for if an increased maximum life span is to evolve is the loss of functioning parts. Such loss may involve any working part of the body, from nonliving connective tissue components, to individual cells or even organs (e.g. thymic involution)—and finally and most importantly involves the loss of working parts of individual cells, including their genetic information stores. In Fig. 2 these sources are depicted and their relative importances estimated. A different and more detailed definition of possible sources of aging was described in an earlier publication (Strehler, 1959).

All specific age changes result from changes that fall into one of the above categories. To the degree that damaging events in one of these categories were sufficiently great that they limited the lifespan of man's ancestors, the evolution of countering genetic qualities must have intervened in order for lifespan to reach its present maximum. These evolved counteracting measures take just four different forms* and probably differ between short- and long-lived species.

A. Evolution of *increased resistance* of individual components to damage;
B. Evolution of means to *increase* the *redundancy* of components that are susceptable to damage;
C. Evolution of means to *decrease* the *concentration* of agents that generate damage;
D. Evolution of mechanisms to increase the *effectiveness* with which damaged components are *repaired.*

There is little tangible evidence bearing on the importance or lack thereof of the first two

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*It is important to realize that there is no a priori reason to believe that the age related changes that limit the lifespans of short lived species are necessarily identical to those that limit long-lived species' life spans. More precisely stated, vinegar eels, fruit flies and mice may well (and probably do) age and die for quite different limiting reasons than man does because the structures and functions that fail and limit longevities may well be quite different from those that fail in aging man. It is precisely because our long-lived species had to evolve damage-counteracting measures (that the mouse may well never need) that our fundamental aging processes may differ from theirs (in regard to life span limitation). If the counteracting measures man has evolved compensate very efficiently for aging defects inherent to, but not life-limiting in, mice then such defects will not appear in aging men to a harmful degree. Instead a "new" set of limiting functions/structures will express themselves during the latter part of the lives of men. The conclusion is that there is no way to know beforehand whether the understanding of mouse, fly or rat aging will shed any valid light on what limits human lifespan and causes human aging. This fact is implicitly accepted by most researchers who use animal "models" of aging (it is cautiously stated by them that results obtained using such models do not necessarily apply to humans). Differences in the factors that limit the lifespans of mice and men are not inconsistent with the well established generalization that all mammals have very similar, though not identical, body plans, metabolic patterns and tissue structures. On the other hand, it seems quite likely that animals that possess similar life spans will age for quite similar reasons, essentially because they have had to solve the same kinds of problems of failure. This would be particularly true when the species being compared are closely related evolutionarily because, in that case, they share near common ancestors that have had to solve at least some of these intrinsic life-span limiting problems.

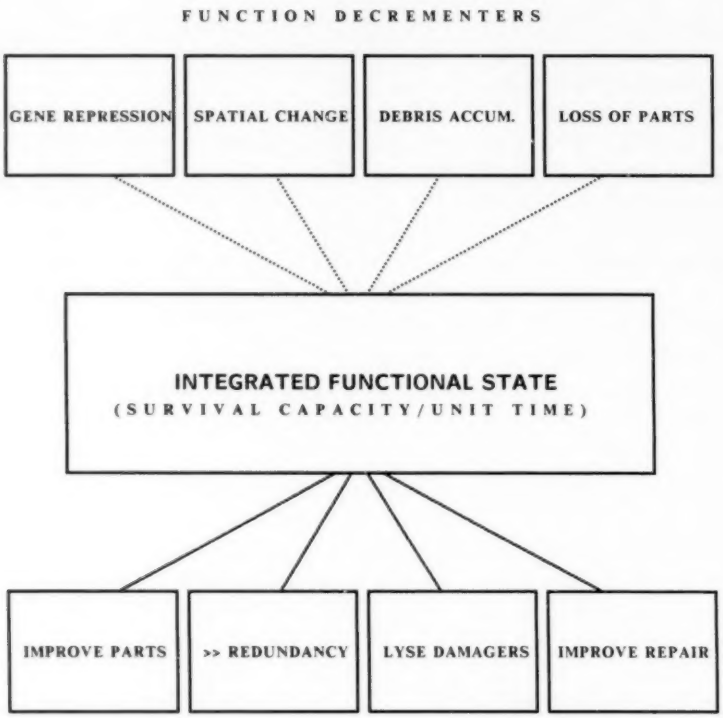

FIG. 2. The top line of boxes shows the key factors that result in a decrease in viability during aging. The effects of the events depicted in these boxes are to decrease the integrated functional state and thus to make the individual increasingly vulnerable to challenges to continued existence (increased mortality rate). The row of boxes at the bottom of the diagram depict the major factors that have evolved so as to counteract or compensate for lifespan limiting damage. See text for further discussion.

categories of such anti-aging compensatory evolution. This writer is not aware of any evidence that there is greater redundancy (except for size increases) of key body parts in long-lived mammalian species than in short lived ones; nor is there convincing evidence that the proteins, nucleic acids, fats or carbohydrates present in short lived species are, per se, more susceptible to damage than are molecules with analogous functions in man. A possible exception is the fact that age pigments evidently accumulate more rapidly in rodents and lower primates than they do in man, but this probably reflects a higher concentration of damage-inflicting molecules in short lived forms than in longer-lived ones rather than a greater susceptibility to damage of individual macromolecules, per se, in short lived species.

By contrast, there is a small amount of evidence that suggests that long-lived species are better able than short lived ones to counteract the effects of damaging substances or agents,

e.g. free radicals or UV irradiation as reflected in "unscheduled" DNA repair (Hart and Setlow, 1976). In the case of unscheduled (UV induced) repair of DNA it must be kept in mind that the greater level of incorporation of tritiated thymidine into the DNA of long lived species than into that of short lived ones during repair does not necessarily mean that repair is more efficient in the former. Alternatively, it could simply mean that the damage inflicted on long-lived species' cells is greater or that larger segments of DNA are removed and then replaced in long-lived lines of animals than in short-lived ones.

Free radicals are believed to be particularly destructive agents. The most common of these ubiquitous substances are the radicals .OH and .OOH. The first of these is derived from hydrogen peroxide (HOOH) when half of the molecule is reduced to water by electron donors such as ferrous ion (Fe^{++}). The iron is oxidized to the ferric state, Fe^{+++}, while half of the peroxide is reduced to water by the addition of an electron (plus a proton) to one of the oxygens:

$$HO\text{-}OH + Fe^{++} + H^{+} \rightarrow HO. + HOH + Fe^{+++} \text{ (Fenton's reagent).}$$

The other damaging substance, HOO. can be produced either by oxidation of an HOOH molecule through extraction of a proton plus an electron thus:

$$HOOH + OXidant \rightarrow H\text{-}OXidant + .OOH;$$

or, alternatively, through partial reduction of molecular oxygen, thus:

$$.O{:}O. = H\text{-}REductant \rightarrow .O{:}Oh + .REductant.$$

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Both the peroxide radical (HOO.—which is called Superoxide in its ionized form, .O:O:-) and the .OH radical are extremely reactive species, much more so than the parent molecules, peroxide and oxygen. This reactivity results from the fact that the unpaired electron present in a radical is able to extract electrons from potential donors, e.g. many different kinds of organic molecules. Unsaturated fats are particularly susceptible to such attack, because of the labilizing effect double bonds have on nearby hydrogen-carbon bonds. Probably equally or even more important than the effects free radicals have on lipids are their effects on the genetic material itself. It is well documented that the mutations induced by treatment with ionizing radiation are greatly increased in number at a particular dose if oxygen is present in the medium in which the organisms are irradiated. This is taken to mean that HOO. radicals are particularly effective in causing damage to DNA.

The most easily visible evidence on the effects of free radical action during aging is the accumulation of the lipofuscin age pigments. These auto-oxidized protein-lipid particles almost certainly are the residues of damaged intracellular membranes (Strehler *et al.*, 1969). They can even be caused to appear in tissues that ordinarily do not contain these substances (e.g. rat uterus) if animals are reared on Vitamin E deficient diets (Gedigk and Fischer, 1959). [Vitamin E is a naturally occuring antagonist to free radical damage because it is able to combine with free radicals and thus to break the self-perpetuating chain of reactions in which a free radical attacks an oxidizable substance (e.g. an unsaturated fat), extracts a hydrogen from it to form a nonradical out of the original radical plus a new organic radical. This latter radical is even more reactive toward oxygen than the original lipid and thus leads to a cycle of perpetuation of radical mediated reactions.

An effect of Vitamin E (and similarly of selenium) is to combine with reactive radicals without producing a new product that perpetuates the chain of reactions.]

A reactive species such as .OH or .OOH is able to react with virtually any organic substance with which it comes in contact. Many of these molecular encounters do not cause any lasting damage to the system as a whole; but others are potentially lethal and their additive effects are probably a very important cause of age decrements in function. In particular, the extraction of hydrogen atoms or electrons from DNA has very great potential effects on the subsequent function of that DNA—if the damage is not repaired.

Such chemical damage to DNA can result in changes either in the coding capacities DNA has or in subtle or gross changes in its structure. Because the information in a DNA double helix is redundant (two copies of the same information—complements of each other are present, one in each of the two DNA strands), there is an opportunity to repair base sequence damage with at least a 50% probability of restoring the original structure. Thus, if a difference (noncomplementarity) is detected (by the error-detecting machinery) between the sense and non-sense strands at a given location, the excision of one or another of the mismatched strand locations followed by replacement of the missing segment through synthesis-repair will have a 50% chance of undoing the damage (depending on whether the damaged segment or the undamaged one was removed and replaced).

The most straightforward means that a biological system can develope to protect its structures from these substances is to evolve a means to convert them to less harmful substances. There exists, in both plants and animals, a number of enzymes that carry out this protective role. The first of these is the enzyme catalase, which converts 2 molecules of peroxide to 2 molecules of water and an oxygen molecule, thus:

$$HOOH + HOOH \rightarrow HOH + HOH + O_2$$

In this dismutation reaction, the two hydrogens on one peroxide molecule are inserted between the 2 oxygens on the other molecule.

The second kind of enzyme is called Superoxide Dismutase (S.O.D.). Its function is to cause one superoxide radical (ionized HO_2.) to reduce another to peroxide while it is oxidized to relatively harmless oxygen, thus:

$$2H{:}O{:}O. \rightarrow 2\,H^+ + 2\,{:}O{:}O. \rightarrow -{:}O{:}O{:}- \,(+2H^+ \rightarrow HOOH) + .O{:}O.\ \text{(oxygen)}$$

Other biomolecules than catalase have the ability to convert peroxide to water and oxygen. For example, hemoglobin (or even hemin) is able to catalyse this dismutation—which is the reason that a solution of peroxide foams when it comes into contact with a bleeding wound.

The effectiveness of catalase and S.O.D. in protecting the body against damage by the chemicals that they dismutate does not depend primarily on the amount of these enzymes present, but rather on the affinity (as reflected in their dissociation constants) that they have for their substrates. Very little additional protection is afforded by producing very much larger amounts of these enzymes than is needed to reduce the damaging molecules to low levels, because such enzymes, regardless of their concentrations, will only reduce the concentrations of their substrates to about the value of the dissociation constants of the enzyme-substrate. Instead, an important evolutionary benefit can be derived if a new mu-

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tant enzyme has a higher affinity for such a substrate. The defense against free radicals, then, does not derive primarily from the quantity of such enzymes present (above a minimum level) but the qualities the enzymes possess. Some workers (e.g. Cutler, 1979) have emphasized the protective effect of large S.O.D. concentrations in long-lived animals (although the data on which this is based is controversial). Such conjectures ignore the basic point that it is the concentration of the damaging molecule that determines the rate of damage.*

A second, still unexplained, effect of mutagens, including the above-mentioned free radicals, is the hydrolysis of the linkages between successive nucleotides in DNA. When such a lesion happens it usually occurs as and is called a single strand DNA break. There is evidently a monitoring system in higher eukaryotic cells that is able to detect such scissions, label them and then to repair them. This system depends upon the activation of a specific enzyme by the physical structure unique to a break, followed by the attachment of adenosine diphospho-ribose (ADPR) to the surrounding protein structures—perhaps as repair "labels". In fact, because NAD is the source of the ADPR moeity, severe damage to DNA (as single stranded breaks) may completely deplete the NAD reserves of a genetically damaged cell to a degree that no recovery occurs—the cell dies because it does not have sufficient NAD to sustain NAD-dependent metabolism.

EARLY WORK ON DNA DAMAGE DURING AGING

The possibility that damage to DNA in somatic cells is a primary cause of the observed age-dependent decline in function was proposed almost 40 years ago (Henshaw *et al.*, 1947). The initial concepts were that somatic mutations in "structural" genes—genes that specify particular products and which are present in only one copy per haploid chromosome set—are the cause of aging. This primary concept does not appear to be consistent with evidence derived since then mostly because (1) the rate of aging of individuals of a species does not depend on the number of copies of individual structural genes contained in its cells (i.e. its ploidy) and (2) because the dose of mutagen required to shorten lifespan measureably is many time greater than the dose of mutagen required to produce a lifetime's worth of "classical" somatic mutations. This last is a particularly key fact and observation.

For these reasons the existing evidence is strongly contrary to the simple mutation

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*In order to examine the possibility that the rate of damage by radicals is lower in long-lived animals than it is in short lived ones because of differences in the rates of destruction of free radicals and their source molecules, such as peroxide, the following previously unpublished preliminary experiments were conducted: Catalase was isolated in semi-pure form from horse heart and from rat heart. To separate solutions of these enzymes, were added identical amounts of hydrogen peroxide. At successive times after this addition, samples were removed from each incubation mixture and injected into solutions of $Fe(CN)6^{+++}$ plus di-methyl bi-acrydylium nitrate or 3-amino phthalic hydrazide, substances that produces light in the presence of the .OH radicals generated by the interaction of ferricyanide and peroxide. An idealized representation of the time course of the disappearance of peroxide in such experiments is shown in Fig. 3. Note that the final level of HOOH present after treatment with Horse catalase is about 3 times lower than is achieved by the rat catalase. This provides tentative evidence that nearly identical enzymes present in long-lived species (as compared to short lived ones) have appropriately enhanced (and presumably greater protective) properties. In order for the general implications of such experiments to be applicable to the problem of the evolution of damage-reducing catalysts as longer-lived species evolved it is obviously necessary that the study be extended to include a much larger set of intra specific comparisons.

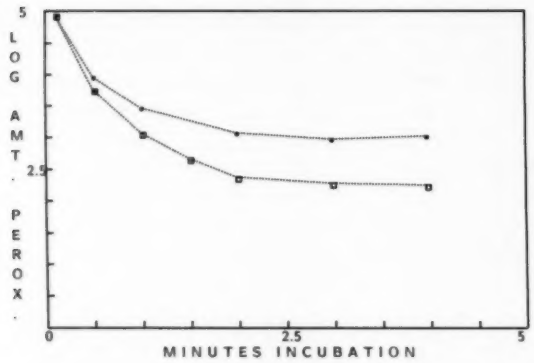

FIG. 3. This figure (arbitrary scale) shows that the relative level to which peroxide is decreased by the enzymes present in horse heart (square boxes) is substantially less (ca. 3 fold decrease) than the asymptote approached when the peroxide lysing enzyme is derived from rat heart. This finding implies that it is not the amount of a given kind of enzyme present, but rather its affinity for its substrate that determines the level to which this harmful agent (peroxide) is reduced and that longer lived mammals reduce this ambient level to substantially lower levels than does the homologous enzyme present in a short lived species (Strehler, unpublished experiments).

theory of aging—the theory that the loss of function of single copy genes coding for the specific amino acid sequences present in proteins is a significant cause of aging of mammals. Instead of mutations to individual genes another kind of genetic damage must be involved—if changes in DNA contribute importantly to the decline in function that characterizes aging.

A listing of the kinds of changes that are possible in the genetic materials includes the following:

1. Point mutations (local, small, deletions or base substitutions);
2. Translocations and transpositions (the transfer of a segment of DNA from one location to another, e.g. from one chromosome to another);
3. Inversions (the removal of a segment of DNA and its reinsertion into the same location, but in reversed order);
4. Double stranded DNA breaks (these result in the appearance of fragments of chromosomes or circular chromosomes, if "healing" occurs);
5. Insertion of nonchromosomal DNA (this may involve the incorporation of "foreign" DNA such as viral DNA into chromosomes. It appears to occur when certain oncogenic viruses are inserted into a host cell's DNA);
6. Deletion of single copy segments of DNA (removal of moderately long pieces of DNA from chromosomes, including whole genes or parts of such);
7. Single stranded DNA breaks (this does not necessarily result in visible changes in chromosomal structure, but would be expected to interfere with gene transcription, if unrepaired);
8. Deletion of tandemly multiple copy gene sequences (removal of one or more copies of DNA segments that specify the same product and which copies follow each other serially).

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The first seven of the eight kinds of damage to DNA listed are either proportional to the amount of damage generated by a mutagen such as ionizing radiation or to the square of that damage (two hit mechanisms). In the case of (single or double stranded) chromosome breaks and point mutations only a single "hit" is required. By contrast, translocations, inversions, insertions and single copy segment deletions require at least 2 such "hits". The latter kind of damage will therefore increase as the square of the dose of DNA-fragmenting treatments (provided that repair processes do not intervene).

The single remaining potential source (out of a total of eight possibilities) of gross DNA damage involving multiple breaks (the loss of one or more copies of tandemly duplicated genes) unlike the other kinds of DNA damage, does not require any double stranded breaks to occur as a result of the mutagenic agent's action. Such deletions are well documented in the case of both the Bar mutation and bobbed mutation reversions to the wild type in *Drosophila melanogaster*. Moreover, deletions of portions of such duplicated segments of chromosomes during meiotic pairing occur because the basic requirement for crossing over between two segments of DNA is that the two DNA regions that will engage in crossing-over must possess essentially identical base sequences capable of being aligned with each other. Following such alignment, the successive occurrence of a pair of single stranded nicks at different locations evidently provides the means through which enchange is mediated and takes place.

A very similar mechanism presumably operates during the insertion or deletion of gene-containing segments of DNA (called episomes) into prokaryotic DNA where the beginning and ending segments of the episome are identical to the sequence at the locus of insertion. Similarly, the transposon-like DNA regions studied in eukaryotes such as Drosophila evidently make use of similar identities between insertion sequence locations and locations to which such "jumping genes" can move. The insertion of viral oncogenes into host cell DNA also involves similar sequence homologies at the point(s) of insertion or deletion. Figure 4 summarizes the above kinds of DNA age changes, their probable relative importance in aging and some inter-relationships between them.

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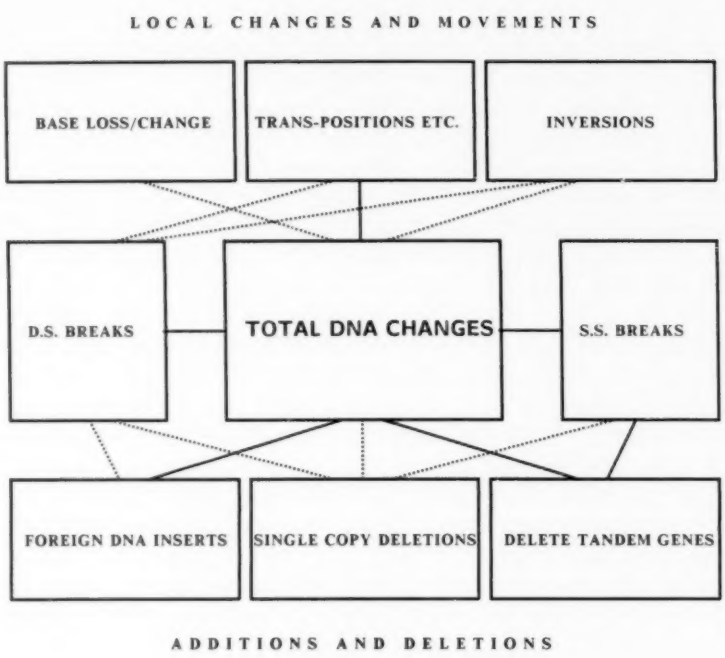
To recapitulate up to this point, the process of aging results from some particular mixture of four alternative classes of processes. The first alternative is programmed processes in which the sequence of specific gene expressions and repressions during development either sets the stage for later deteriorative changes of many different kinds or, in some instances, directly "causes" these changes (e.g. as in the sudden senescence of Salmon). The second alternative process is that aging reflects the effects of either "classical" gene mutations' accumulation in somatic cells or of some more subtle kind of change in DNA. The third possibility is that aging is the cumulative consequence of internal and of environmental factors that affect the above two processes and/or produce random disruptive changes such as the accumulation of cross-linked lipofuscin age pigments (Strehler *et al.*, 1959). The final is that random rearrangements of body, cell and tissue parts are responsible.

If the aging process were primarily the result of the expression of a genetic program then one might ultimately expect that the development of means to manipulate gene expression would permit a modest or even major control/reversal of some of aging's effects – provided that special drugs, diets or viral carriers of modulators of gene expression that could affect the program were developed and used. If senescence were to a significant degree the result of modifiable environmental factors, then its amelioration might also be expected, to some degree. But, if aging is the secondary effect of the accumulation of



AGE CHANGES IN DNA

FIG. 4. This diagram shows the relationships between the status of DNA as a function of aging and the various kinds of damage and rearrangements that DNA can suffer during the passage of time. The dotted lines indicate a probably relatively small contribution to total DNA alterations; the solid lines indicate probable large effects. D.S. Breaks means Double Stranded Breaks (i.e. complete severance of the continuity of a DNA molecule) whereas S.S. Breaks denotes cleavages of bonds in only one DNA strand, thus leaving the molecule partly intact. The dark lines include particularly (A) transpositions (such as the rearrangements that occur in the gene sequences that determine antibody forming capacity or the transposon-like segments of DNA that evidently may move from place to place in the genome during development and aging); (B) the insertion of foreign DNA such as takes place when an oncogenic viral sequence is incorporated into a host's DNA; (C) the loss of parts or complete segments of genetic material that occurs in multiple (tandem) duplications, one after another in the chromosomes of higher forms of life. These losses are probably ascribable to events consequent to single stranded breaks in DNA; (D) because of the large number of chromosomal breaks that occurs during aging as documented by Curtis and his colleagues, double stranded breaks in DNA are given a heavy emphasis, although their effects on function are unknown. The other kinds of damage are considered as of lesser importance, particularly because agents such as mutagens do not seem to decrease the efficiency of DNA function in proportion to the increase in such damage—a key deduction dealt with in the text.

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genetic damage, then the prospect for its early modification is not particularly promising, largely because altering aging would require the development and installation of complex and new means to repair accumulated genetic damage. In the remainder of this paper evidence is presented that genetic damage (particularly gene loss) is almost certainly a (or probably the) central cause of aging.

As discussed earlier, genetic damage occurs in many different forms. These include the alteration or substitution of single nucleotides, the deletion of single or short segments of nucleotides, DNA fragmentation, masking of DNA by histones or other proteins or other adducts, the inactivation of specific genes, rearrangements of segments of DNA so as to render them nonfunctional, and finally the impairment of key cellular functions due to the loss of complete, specific, DNA sequences.

Alterations in DNA, following its synthesis, also include the loss of amino groups from cytosine and from adenine and the methylation of certain cytosines when they are in particular guanine-containing sequences. Some, highly tentative and controversial evidence exists that proteins or other substances may form significant numbers of cross-linkages between DNA strands during aging (Cutler, 1975). The nature of these substances (except for some particular synthetic chemical mutagens or thymine dimer formation) is not well established and despite much speculation on this point there is no compelling evidence that age-related cross-linkages in DNA exist in vivo to any measurable degree. By contrast, the formation of thymine dimers consequent to the ultraviolet irradiation of DNA is a major cause of damage in cells exposed to UV radiation and this phenomenon is the basis of studies on "unscheduled" DNA synthesis or repair (Hart and Setlow, 1974).

To return to the possible relationship between mutations and aging, this possibility was first pointed out by Paul Henshaw and his co-workers (Henshaw *et al.*, 1947). They showed that rodents exposed to nuclear pile irradiations exhibited some external signs of aging (e.g. greying of hair) and, provided that the exposure was sufficiently high, the animals also exhibited life-shortening. Sacher at the Argonne National Laboratory (Sacher, 1956) obtained results generally consistent with the above, except that there was some indication that moderately irradiated animals often showed an increase in longevity. At a meeting in 1957 in Gatlinburg, Tenn., sponsored by the AIBS-NSF (Strehler *et al.*, eds., 1960) there was a wide-ranging discussion of the pros and cons of mutations as a primary cause of aging. Failla (p. 170), previously a proponent of the mutation theory, there concluded that mutation could not be a major cause of aging because the dosage required to shorten life was about 12 times greater than that required to double the mutation frequency over the control rate (in mice). The author (see page 57, 1960) at that time suggested that insects such as Habrobracon, which can exist in either the haploid or diploid form as imagos, were suitable organisms for a test of the hypothesis that harmful recessive mutations are responsible for normal aging—because, if such recessive mutations cause aging the life-spans of haploids should be shorter (in the absence of mutagenic agents) than those of the diploid forms. Clark and collaborators, using Habrobracon, (Rubin and Clark, 1961) shortly thereafter tested this prediction and showed that although the haploids were substantially more sensitive to x-irradiation than were the diploids (as regards life shortening), the normal life spans of these two genotypes under laboratory conditions were essentially identical independently of ploidy.

This observation seemed to rule out mutations as a major cause of aging, at least in that species (See Fig. 5). Nevertheless, during the following decade, Howard J. Curtis and his colleagues conducted many imaginative and critical experiments which appeared to give support to the mutation theory of aging (see Curtis, 1966). The essence of Curtis' findings were (A) that the percentage of (liver) cells that showed visible signs of genetic damage (chromosomal aberrations of various kinds) increased from about 5% in newborn rodents to 24% in two year old animals; (B) th. the number of such aberrations was markedly increased by exposure to various kinds of mutagenic radiation; (C) that the damage induced

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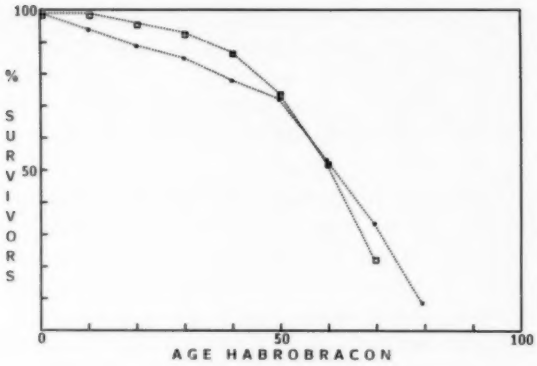

FIG. 5. The longevity of the wasp, Habrobracon, is not affected by the number of copies of chromosomes it contains. The small squares correspond to the survivorship vs. age of diploid (two copies of each chromosome) whereas the open boxes depict the survivorship curves of individuals that have only a single copy of each kind of chromosome. The significance of this finding by Clark and his collaborators is that aging, in this species, cannot be caused by spontaneous recessive mutations that decrease function because there is no significant decrease in longevity of haploid individuals compared to diploid (2 copies of DNA) ones (replotted after Rubin and Clark, 1960).

by x-radiation (but not by neutrons) appeared to become "repaired" with time's passage; (D) that the phenomenon took place more rapidly in short lived strains of mice than in long-lived ones; and (E) that the rate of aberration accumulation was substantially slower in dogs than in rodents. Particularly key aspects of Curtis' findings relevant to this discussion, are summarized in Fig. 6.

Curtis' monograph (Curtis, 1966) succinctly presents these finding and a concise and somewhat persuasive interpretation of them. However, in an invited review of Curtis' book in *Science*, the present writer pointed out that the neutron doseage required to produce chromosomal damage equal to that accumulated during the lifetime of the animals was only 67 roentgen equivalents, and that this dosage that produces no measurable life shortening. It was reasoned that the kind of mutations measured (and similarly for so-called "point mutations") could therefore not be responsible for "normal" aging in the species studied. An important caveat was added, however, as follows: "Should some other kind of mutational event (not of the classical kind) occur at a high frequency under control conditions and not be increased in proportion to the classical genetic damage induced by the mutagenic treatment, then such hypothetical genetic damage, if it occurred at all, could in fact be a (or the) cause of aging."

In order to test the possible validity of this caveat we began our studies on the possible instability of tandemly duplicated parts of the DNA. As pointed out earlier, it had long been known that the Bar locus in *Drosphila melanogaster*, a tandem duplication in the X chromosome was very unstable genetically. Asymmetric pairing during meiosis evidently occurs in about 5% of all cross-overs in this region, leading to hyper-Bar individuals (3 tandem copies) and equal numbers of normal wild type flies with no such duplication. K.C. Atwood had also noted a similar instability of the Bobbed mutation in the same organism and that this mutation co-mapped with the so-called nucleolar organizer. It was

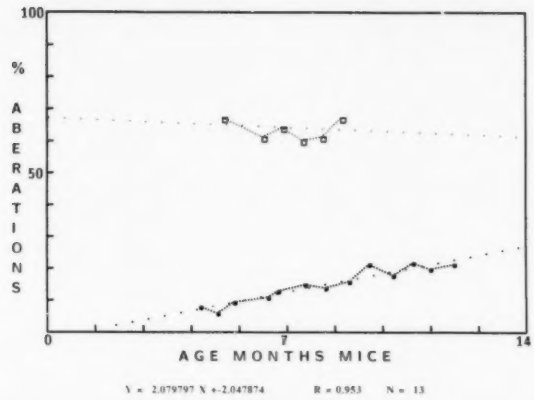

FIG. 6. Two important observations by Curtis and his co-workers are shown. The first, shown in the lower curve, is that the percentage of cells that possess at least one visible chromosomal aberration in the livers of mice increases as the ages of the mice increase. The number of such aberrations (chromosomal breaks, circles, etc.) is essentially 0 at birth and increases at the rate of about 2% per month thereafter, so that by two years of age about 46% of liver cells would be expected to have incurred such damage. This observation, taken by itself, would tend to substantiate the thesis that aging decrements in function are caused by chromosomal aberrations or by local mutations in structural genes, which are believed to occur proportionally to gross chromosomal damage. However, the upper curve (large squares) shows the effect of the relatively small dosage of ~192 roentgen equivalents of neutrons has on the same kind of chromosomal damage. More than 60% of the cells in mouse livers show abnormal chromosomes after being treated by this level of mutagenic radiation. This, in turn, means that a full lifetime's worth of such genetic damage is caused by about 100 roentgen equivalents of neutrons. It is because this amount of radiation does not produce instantly "aged" mice that it is most unlikely that either chromosomal aberrations or specific gene mutations are an important cause of aging. This evidence, plus that presented on Habrobracon (Fig. 5) make any simple mutation theory of aging untenable. (Y = 2.079797 X + −2.047874; R = 0.953; N = 13.)

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partly upon this basis that experiments involving rRNA-rDNA hybridization were undertaken by Ritossa, Spiegelman and Atwood (1966). Their experiments (See Fig. 7) clearly demonstrated that the severity of the bobbed phenotype is a direct function of the amount of tandemly duplicated rDNA lost in such mutants.

STUDIES ON rDNA LOSS DURING AGING

On the basis of these prior concepts and work we undertook to determine whether the rDNA dosage of older mammals resembles the bobbed mutation, a proposition which was not considered likely by us or others but which could lend a new credence to the essential hypothesis of Curtis and others—that genetic damage is a (or the) major cause of aging. Accordingly it was arranged with Dr. D. Chrisp of the AEC laboratory at Davis, CA. to obtain fresh autopsy material (initially only brain tissue) from beagle dogs (of various ages) and to measure the rDNA dosage as a function of age using the Ritossa-Spiegelman-Atwood hybridization procedures.

Our earliest results (Johnson and Strehler, 1972), strongly supported the thesis that tandemly duplicated DNA, such as rDNA, is unstable during aging, particularly in post-mitotic

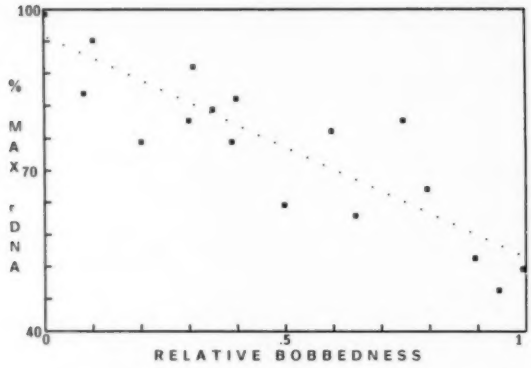

FIG. 7. This representation is based on the relationship that exists between the degree of bobbedness, a specific phenotypic mutation in Drosophila, and the amount of rDNA present in these mutants (Ritossa *et al.*, 1966). The phenotypic difference between normal (wild type) flies and the bobbed mutants is in the length of the bristles present on the adult flies. In the presence of the mutation the bristles are shortened and bent at the top (thus "bobbed"). Mutants with different degrees of bobbedness exist and the evidence indicates that the bobbed character is due to a dificiency of rDNA genes, as measured through molecular hybridization techniques. This should be compared with Fig. 14 in which the kind of relationship found between the degree of bobbedness and lifespan of these mutants is shown. A central thesis of this paper is that aging is essentially a gradually acquired bobbed-like state in post-mitotic cells.

tissues. As a result of these findings (in which yeast rRNA was used as the hybridization probe, because of its nearly identical sequence and low cost of production) the study was expanded to include six different tissues from beagle dogs of different ages (Strehler, Chrisp and Johnson, 1972). No significant age differences were found in replenishing tissues' DNAs (liver, kidney, spleen) but, by contrast, the results on 3 nondividing cell types revealed a quite significant loss as shown in Fig. 8. These Figure panels document a loss in rDNA hybridizability in all post-mitotic tissues tested (heart, skeletal muscle and brain). Because the percentage of loss by age 10 was nearly 35% in the most severely affected tissues and because it is unlikely that each cell in these tissues suffered an identical loss, it follows that some of the cells probably had incurred rDNA losses up to 70% or more whereas other cells probably were less affected. The number of such cells clearly depends on the shape of the cell-lesion-distribution curve, which in turn depends on the average number (unknown) of such "hits" sustained by the cells.

Such a major and presumably irreversible loss of a vital component of irreplacable kinds of body cells as well as the implications that these findings have regarding the chances for significantly modifying the rate of the aging process in the near future was in a subjective sense disappointing. Hopeful skepticism led us, in subsequent confirmatory experiments, to use a quite different measurement and hybridization technique in studies on difficult-to-obtain human heart tissues (supplied by former Los Angeles coroner, Dr. Thomas Noguchi). At the suggestion of Prof. Norman Davidson of Cal Tech, we used hybridization in the liquid phase, followed by selective and separate elution of the double stranded vs. single stranded nucleic acids from a chromatographic column. The first studies on human rDNA dosage vs. age (Johnson *et al.*, 1975) again, as in the case of the work with dog heart, indicated a very substantial loss of rDNA from this human tissue as a function of age.

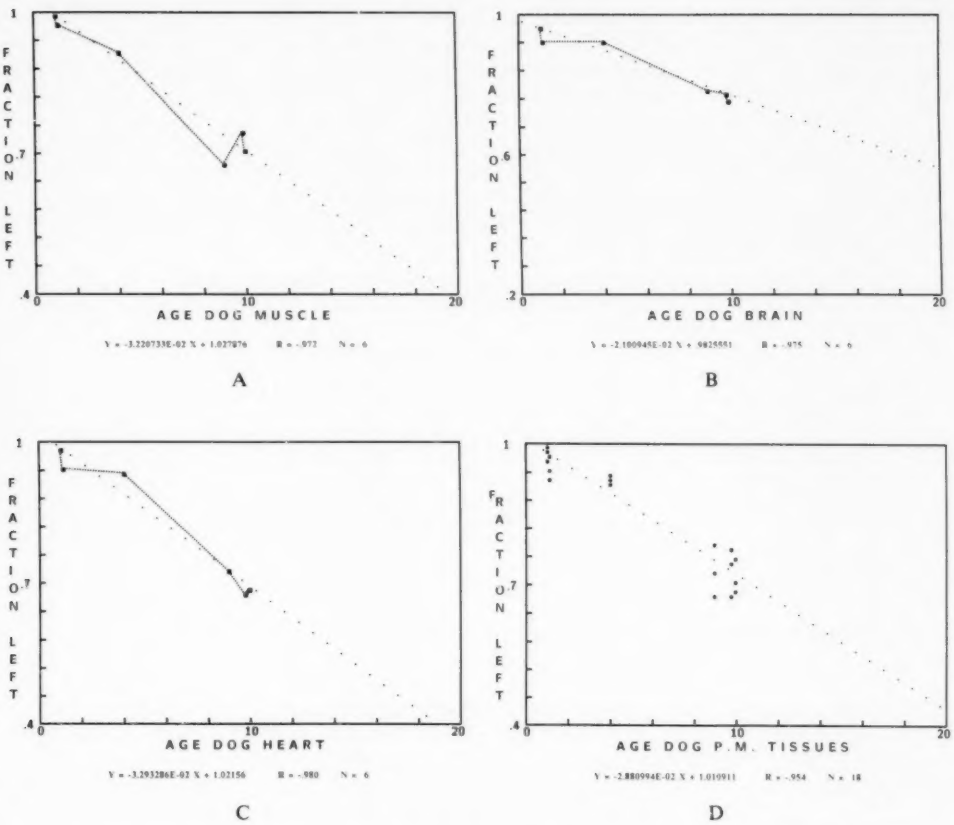

FIG. 8. The four panels of this figure show the measured decrements in rDNA content per unit weight of DNA in DNA obtained from three different post-mitotic tissues of beagle dogs. Panels A ($Y = -3.220733E-02\,X + 1.027876$; $R = -.972$; $N = 6$), B ($Y = -2.100945E-02\,X + .9825551$; $R = -.975$; $N = 6$) and C ($Y = -3.293286E-02X + 1.02156$; $R = -0.980$; $N = 6$) show respectively the rDNA content vs. age of beagle muscle, brain and heart, Panel D ($Y = -2.880994E-02\,X + 1.010911$; $R = -0.954$; $N = 18$) illustrates the decrease in rDNA found when the values for the individual tissues are all plotted on a single graph (data after Johnson, Chrisp *et al.*, 1972).

This study was subsequently expanded to include 29 individual human hearts. The previous results were again validated (Strehler *et al.*, 1979a). The evidence indicates that ca. 0.5% of the original amount of rDNA is lost per year from human heart (see Fig. 9). An even greater rate of loss (ca. 0.7%/yr.) was then demonstrated in two separate regions of the human brain (hippocampus and sensori-motor cortes—see Fig. 10). Moreover, the rate of loss/year is 5–7 times more rapid from dogs' post-mitotic tissues than it is from the same human tissues (see Table 1). This number is very similar to the ratio of the maximum longevities of these two species (ca. 120 years vs. 20 years, a 6/1 ratio)—which implies that this loss limits the maximum lifespan of both species. Table 1 presents a summary of the decremental values found in various mammalian tissues, a comparison of the rates of gene

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TABLE 1. THE RATE OF LOSS OF rDNA FROM VARIOUS TISSUES OF TWO SPECIES, MAN AND DOG IS SUMMARIZED. THE UPPER PORTION OF THE TABLE DISPLAYS THE RATES OF LOSSES OF NOR'S FROM LYMPHOCYTES AS A FUNCTION OF AGE AND THE LOSS OF rDNA VS AGE IN SPECIFIED HUMAN AND DOG TISSUES. THE CENTRAL PART OF THE TABLE SHOWS THAT THE LOSS OF THESE STRUCTURES IS ABOUT 4.5 TIMES MORE RAPID IN DOGS THAN IN MAN AND THAT THE RATIO OF LONGEVITIES OF THESE SPECIES DIFFERS FROM THE ABOVE RATIO BY LESS THAN 20%. THE BOTTOM PORTION OF THE TABLE SHOWS THAT THE RATE OF LOSS OF rDNA IN HUMANS IS QUITE COMPARABLE TO THE RATE OF LOSS OF VARIOUS FUNCTIONS IMPORTANT FOR SURVIVAL. THE KEY POINT IS THAT IF PROTEIN SYNTHESIS IS LIMITED BY THE NUMBER OF COPIES OF rDNA GENES PRESENT AT A GIVEN AGE, THEN SUCH DECREASED PROTEIN SYNTHESIS WILL GENERATE PROPORTIONAL DEFICIENCIES IN THE MAXIMUM RATE AT WHICH SPECIFIC KINDS OF WORK NEEDED TO PRESERVE AN INDIVIDUAL CAN BE PERFORMED.

COMPARISON OF LIFESPAN, FUNCTIONAL LOSS AND rDNA LOSS
(Humans vs Dogs)

TISSUE	SPECIES	%LOSS/YR
Lymphocyte NORS	Man	.45
Brain -- SS Cortex	Man	.79
Brain -- Hippocampus	Man	.80
Heart Muscle	Man	.60
Brain	Dog	2.10
Muscle	Dog	3.20
Heart	Dog	3.30

AV. (Brain, Heart, NORS) Man .61
AV. (Brain, Heart, Muscle) Dog 2.87

RATIO LOSS RATE (DOG/MAN): (2.87/.61) = 4.69

RATIO MAXIMUM LONGEVITY (MAN/DOG) (110/20) = 5.5

RATIO POST-MATURE LONGEVITY (MAN/DOG) (95/19) = 5.0

PERCENTAGE FUNCTIONAL LOSS IN DIFFERENT SYSTEMS -- MAN

FUNCTION	% LOSS/YEAR
Max. Breathing Capacity	1.09
Renal Plasma Flow	1.00
Vital Capacity	0.76
Cardiac Index	0.71
Cell Water	0.46
Nerve Conduction Velocity	0.33
Muscle (HandGrip Strength)	0.66
Incidental Memory	0.89
Androgen Excretion (Males)	0.82

AVERAGE % LOSS/YEAR 0.746

RATIO: AVERAGE FUNCTIONAL LOSS/rDNA LOSS = (.746/.61) = 1.22

loss from dog and human tissues and the proportionality between the rate of various kinds of functional losses during aging in humans and the rate of rDNA loss. The precision with which rDNA losses may be measured through the above-described techniques is illustrated in Fig. 11. The points on this graph show that the level of rDNA is essentially the same in samples taken from two different parts of the brain of the same individual independently of that individual's age.

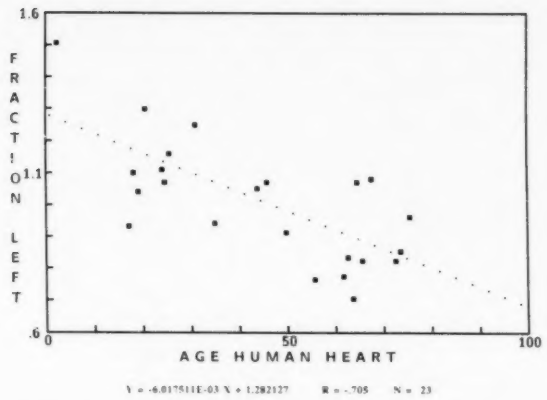

FIG. 9. This figure shows the relationship between rDNA content per unit DNA of human hearts as a function of age of the human donor (autopsy). It should be noted that the variance of the values around the age mean (as defined by a least squares fit to all points and R values) is much greater than was found for beagle dogs which are presumably more genetically uniform than are humans (see Fig. 8). The rate of loss of rDNA in human hearts as determined in these studies is about 0.6% per year. Thus, by age 100 about 60% of the original rDNA would have been lost (after Strehler *et al.*, 1979a) ($Y = -6.01711E-03X + 1.282127$; $R = -0.705$; $N = 23$).

PARALLELISM WITH FINDINGS ON LOSS OF NORs FROM HUMAN TISSUES

There is another line of evidence that is consistent with and probably relevant to the loss of rDNA hybridizability with age. This evidence is based on the presence of NOR's (Nucleolar Organizing Regions) on a number of human chromosomes. These regions are characterized by their selective affinity for silver stains and are believed to signify active regions of rRNA synthesis at these specific chromosomal regions. The number of such active NOR's per cell has recently been shown by two separate groups of workers to decrease monotonically with age in human lymphocytes (Denton *et al.*, 1981; Das *et al.*, 1986). A plot of this data is shown in Fig. 12. The percent loss of stainable NOR's is comparable to (though slightly lower than) the percent of rDNA loss we had reported in post-mitotic human tissues. A slightly lower rate of loss of NORs from human fibroblasts was reported by Buys (Buys *et al.*, 1979—see Fig. 12A). These findings raise the interesting question of whether the disappearance of NOR's with age reflects the simultaneous loss from individual chromosomes of all rDNA copies present on such single chromosomes or whether the loss of NOR's varies among cells derived from a given clone of lymphocytes.* In other words, if it should turn out that the clonal descendents of a given lymphocyte always show the same number of NOR's then the loss would appear to be a permanent event, rather than the product of some subtle kind of regulation of these genes' expressions on some chromosomes but not on others in the same cell.

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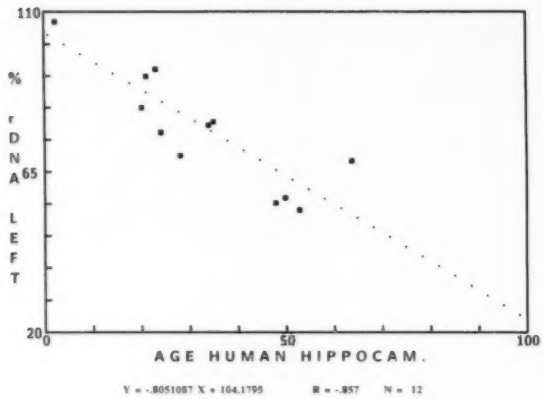
Buys' 1979 publication presents certain evidence that deficiencies in NORs are inheritable. "At least part of these differences seem to be heritable. . . . the differences in silver stainability of chromosomes are conserved after replication. The uniformity in size of silver dots on sister chromatids might . . be taken as an indication of a heritable nature of the silver stainability" (See Buys *et al.*, 1979).



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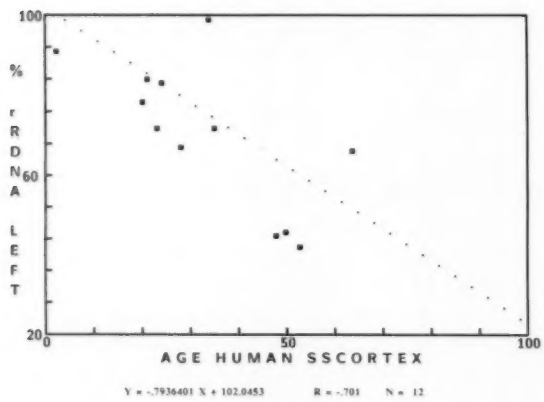

B

FIG. 10. Panel 10A (in which Y = −0.8051087 X; R −0.857; N = 12) shows that the loss of rDNA in the human hippocampal lobe occurs at about 0.8% per year whereas panel 10B (Y = −0.7936401 X + 102.0453; R = −0.701; N = 12) shows an almost identical (0.79%/yr.) rate of loss in somato-sensory cortex from the same group of individuals. Whether the 25% difference between heart and brain observed is significant cannot be determined on the basis of the data available. However, in both cases the loss is highly significant and will result in complete depletion of rDNA by age 130 or 160 (after Strehler *et al.*, 1979b).

OTHER rDNA STUDIES

In 1975 Gaubatz measured the rDNA content of DNA vs. age of a small number of animals using a new and untested method he had developed to isolate and purify DNA. In his earliest publication (Cutler and Gaubatz, 1975) some evidence was reported for rDNA loss. However, shortly thereafter Gaubatz reported that he could confirm neither our results nor their own earlier ones (Gaubatz *et al.*, 1976). In a somewhat later personal communication (Cutler, 1977) it were confirmed that rDNA is indeed lost from "very aged ani-

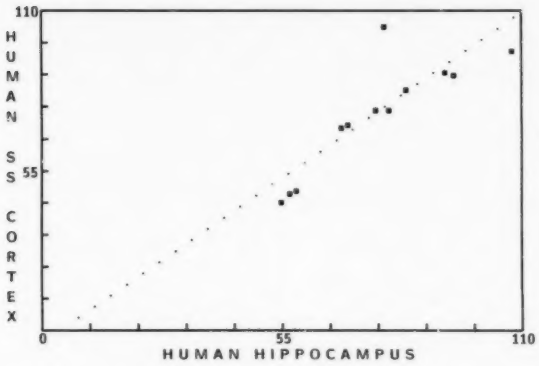

FIG. 11. This figure shows that the large variations between rDNA contents of cells from different individuals of about the same age are not the result of measurement errors. The nearly straight line generated by the points on the diagram (in which the amount of rDNA measured in the hippocampus of an individual was plotted against the amount of the same substance measured in a sample from a different brain area of the same individual) indicates that separate samples from the same kind of tissue give essentially identical measured values of rDNA content. (Y = 1.03266 X −4.271642; R = 0.857; N = 12)

mals" (ca. 1200 days), but that he found little or no change between 1 and 2 year old animals (rodents) or in the few human tissue samples (ca. 10 individual tissue samples) examined.

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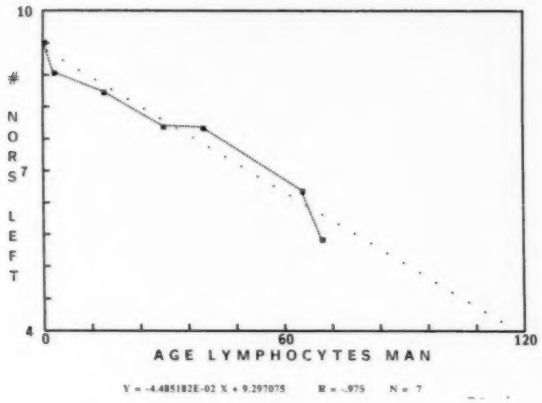
In the following year these experiments were published as part of a review (Cutler, 1978). Significant decreases in rDNA hybridizability was reported in the above-mentioned mouse tissues. However, in the same paper Cutler presented an experiment which he interpreted to mean that some kind of undefined "cross linkage" exists in the DNA from old cells and speculated that this explains the reduced hybridization of old DNA with the labeled probe. The basis for this controversial and unverified claim is that treatment of a single sample of DNA from an old animal studied, with a reducing agent and chelating agent, plus alkali, plus denaturation of the DNA plus retreatment with pronase yielded a higher hybridization values than a control that had been carried out during a different earlier experiment. (In fact the rDNA content of this single sample was higher than any of the dosages of this gene found for DNA from young animals in previous experiments from that laboratory.)

There are several reasons to question the validity of any conclusions based on such a single experiment on a single DNA sample. The first of these is that the "control experiment" could not have been carried out at the same time as the hybridization with the "retreated" DNA. This is made evident through a study of the graph presented in an early part of that review paper and the table that purports to show a change in hybridizability of DNA as a result of treatment. The hybridization figures for the control experiment (untreated DNA) are identical, point for point, to those plotted in an earlier section of that paper. The author states that the results on the 12 DNA samples taken from 3 different animals are consistent with our previously reported findings and that for this reason it was decided to find out whether the kinds of protein-DNA cross linkages he had earlier theorized (Cutler, 1975) might be the cause of the differences between DNA from different aged animals. The drastic and potentially destructive multiple treatments of one spleen DNA sample with the



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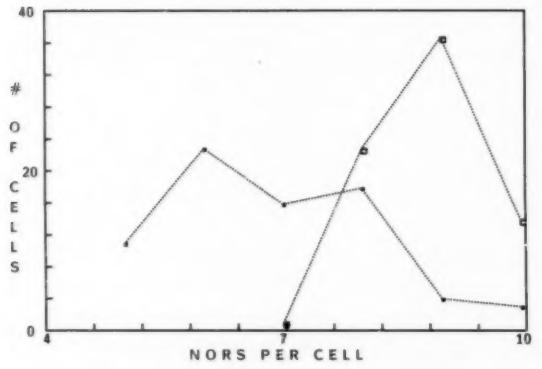

B

FIG. 12. In Fig. 12A (in which Y = −4.485182E − .02X + 9.297075; R = −0.975; N = 7) the combined results (in studies of lymphocytic NOR's) of two different groups of investigators are plotted as a function of age. The ordinate shows the average age of the group from which the average numbers of Nucleolar Organizer Regions (NOR's) were obtained and the abscissa shows the number of NOR's observed. The points corresponding to ages 0, 3, 30 and 65 are derived from the work of Das (Das *et al.*, 1986) and the remaining values from Denton's studies (Denton *et al.*, 1981). The rate of decline of stainable NOR's is quite comparable with the total rDNA content decline found for other tissues. In Fig. 12B, the effect of age on the number of NORs per fibroblast is shown. The right-hand curve shows the distribution of numbers of NORs in fibroblasts obtained from a small sample of humans under 1.5 years of age whereas the left-hand points indicate what was found when fibroblasts from individuals over 80 years of age were examined. It is clear that, with age, there is a loss of stainable NORs. These data are plotted from the values given in Tables V and VI, p. 66, of Buys *et al.*, 1979. A similar loss of NORs in other tissues may be responsible for the decrease in rDNA content described in Figs. 8–10. If the favored location for crossing overs were the terminal regions of NORs, then the result would be the complete removal of the entire tandemly duplicated train from a given chromosome. Such events could well account for the loss of stainable NORs vs. age described by other workers. A crucial experiment needed to test this possibility is to determine whether the descendents of a given lymphocyte or fibroblast (a clone) have essentially identical numbers and kinds of NORs.

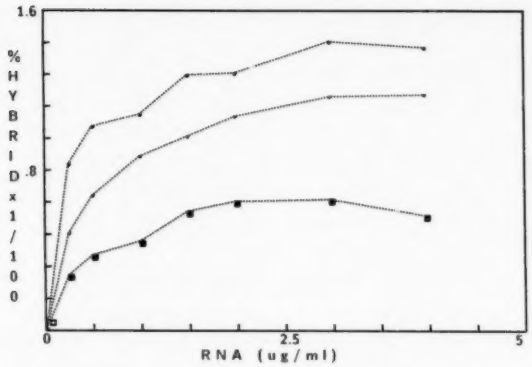

FIG. 13. This describes the results reported by Cutler (Cutler, 1978). The lower curve shows the amount of labeled rRNA bound per unit DNA from a mouse spleen of age 905 days. The squares show the values reported in Table 9, p. 484 (Cutler, 1978). The small square within the upper left corner of each of these points is the value extracted from Fig. 4 (bottom right) of that same paper. The upper curve shows the purported hybridization values obtained after the same sample as shown in the lower curve was "treated." The middle curve shows the values obtained for rDNA from the same tissue when the donor animal was only 40 days of age. From these values it can be concluded with certainty that the upper curve and the lower one could not have been generated at the same time and that the difference between them is due to differences in conditions of incubation or to differences in the reagents employed. The middle curve shows that the youngest individuals tested had ostensibly lower rDNA values than did the "treated" DNA from an old individual mouse. It follows that if the control experiments were inadequate, then the results as a whole are not true measurements of the variables and therefore that no valid conclusion can be drawn from this single experiment.

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above combination of agents resulted in a new set of hybridization values reported in a separate table in that paper. However, because some (unknown amount of) time must have elapsed between the first set of experiments and their evaluation and the subsequent treatment and hybridization of the single 905 day old spleen DNA sample used, it is evident that the reported untreated "control" could not have been carried out at the same time, with the same labeled reagent (RNA) and perhaps not under identical conditions of hybridization as was used on the experimental "treated" DNA. It is therefore completely incorrect to conclude that the treatment affected hybridization in any way whatsoever (See Fig. 13 and legend).

The second reason for doubting the reliability and significance of this single measurement on a single DNA sample is that the hybridization values obtained with a given labeled rRNA sample are notoriously variable if the labeled probe is stored for any substantial time. In this regard Chang and Strehler (see Strehler and Chang, 1979) have found that the measured hybridization values increase as the probe ages, presumably because of scissions of the probe induced by its own radioactive decay. For this reason, in experiments from our laboratory we have compared the amount of hybridization obtained between 2 or 4 samples of different ages (parallel runs on the same day) so as to compensate for any changes in efficiency in the probe's binding or other unknown variables. Evidently these other workers were not aware of this source of variability in measured hybridization efficiency. It is possible that the large variability reported in the other tables of Cutler's 1978

paper between rDNA content of DNA's from humans of approximately the same age also reflect this kind of potential experimental error (as much as 100% difference between DNA's from persons of almost identical ages was reported). Cutler (Cutler, 1978) has correctly pointed out that the basis of his conclusions needs to be tested on additional samples, but in the meantime no such confirming or denying evidence has appeared.

In summary, although the work of Cutler and Gaubatz appears to confirm an apparent loss of rDNA in those cases where the experiments were carried out at the same time on DNA samples from animals of different ages (e.g., see Fig. 4 (lower right, page 481 of Cutler, 1978), there are compelling reasons to doubt that the single experiment on a single DNA sample reporting evidence of cross-linked proteins present in the old DNA samples has any significance whatsoever. Rather, the weight of the evidence available, in which conditions were carefully controlled and in which the experiments were carried out with 3 different colleagues over a ten year period in our laboratory, indicates that there is a very substantial loss of rDNA genes during aging. Even if it were the case that the hypothetical cross-linkages Cutler believes may exist do exist, such a large blockage of access to DNA through any real cross linkage would be just as effective in blocking rDNA transcription as would be the loss of these genes, per se.

DISCUSSION

In order for any proposed mechanism of a dominant cause of aging disabilities to have validity, the proposed process must possess two qualities: The first of these is that the process proposed must take place gradually and particularly involve post-mitotic cells (because this is where the most striking aging related decrements in function occur); secondly, the defects must be sufficiently large and involve such totally essential cell functions as to be able to account for the decrease in body function that occurs during aging. In humans, this decrease in function is about 0.97% per year (after age 30) occur; according to the seminal cross-sectional studies on many different systems carried out by Nathan Shock and his many collaborators and colleagues. (See Shock *et al.*, 1956, 1959 or Strehler, 1977 for summary of much of this work). The observed loss of rDNA is between 55% and 80% of this rate of functional loss.

The rate at which specialized work can be carried out by a tissue is the difference between the maximum amount of work of any kind it can do and the amount of work required to maintain that tissue and its cells. This difference may be called its reserve power capacity (See Strehler and Mildvan, 1960, for discussion). Thus, some unknown and relatively constant amount (perhaps 10–20%) of the rDNA of a cell is involved in simple cell maintenance and housekeeping. It is only the remaining work capacity that is available to perform those specialized cell functions needed to maintain the system. Therefore, it follows that the decrease in specialized function must be greater than the decrease in rDNA content of a cell. It is for this reason that the loss of rDNA would be expected, as is found, to be less than the decrease in specialized work the cell can do. The proportion of function lost per unit loss of rDNA cannot presently be measured because there is no presently known experimental means through which the rDNA dosage can selectively be decreased in an adult animal by a known amount and then the effects of such loss on specialized function be evaluated.

There is another means through which the effects of partial loss of key genetic components, which the genes coding for ribosomal RNA are, could be evaluated. This follows

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from the fact that it would not be expected that each cell would have an equal amount of loss of rDNA at a given age of the host animal. Equal loss would not be expected of any stochastic process unless the number of events leading to partial loss were very large. In this case, the departure of an individual cell from the mean could be minimal. However, if few events are responsible for the loss of a component such as this in each individual cell, the variation around the mean could be very large indeed [particularly if the number of such events (hits) is very small as proposed by Szilard (Szilard, 1959)]. In such a case if the average difference from the mean loss is 50% of the loss, then a total average loss of 70% could well mean that a significant portion of the cells have lost 90% or more of their original complement of such genes. Evaluating the spread of values within individual cells in a population of cells depends greatly on the hypothesis of mechanism of loss assumed. Because one could choose that hypothesis that fits a particular preconception this is not an appropriate mathematical approach to apply. It is therefore imperative that the variance around the mean of individual cells' rDNA dosage be measured. Perhaps the most straightforward approach would be of determine whether the pattern of NOR activities is constant within different clones of lymphocytes or fibroblasts. If it is, then these deficiencies in staining probably represent essentially complete deletion of rDNA genes from certain chromosomes, presumably on a random "hit" basis.

The fact that two processes (tandemly duplicated gene loss and aging) parallel each other does not of and by itself establish a causal relationship in either direction between the two phenomena. Nevertheless, a loss of 50% or more of such an absolutely essential genetic material as rDNA is very unlikely to be without any effect on the function of any living system. Indeed, in those cells that have lost nearly all of their rDNA it is very likely that such cells' maximum responses to challenges that require rapid (maximal) protein synthesis, will be minimal or absent. Therefore, whatever fraction of cells has lost responsivity completely will decrease the total work capacity of the system proportionately to this percentage of cells rather than in proportion to the fraction of rDNA lost on the average from the cells making up that tissue.

Of very probable relevance is the observation by Mann and Yates [a confirmation and quantitation of an earlier report by Andrew (Andrew, 1952)] of a decreased rRNA content of neurons of old humans (Mann and Yates, 1974). This decrease is almost exactly the same/year as is the rDNA dosage loss we have documented in heart muscle (0.4% vs. 0.47%/yr.) and is quite consistent with the deduction that a lower rDNA dosage results in a lower steady-state pool of rRNA (in neurons, in this case). Because neurons continuously export proteins needed for cell functions from the cell body to the farthest extensions of their axons and dendrites, any substantial reduction in synthesis of any such essential proteins would almost certainly reduce the cells' abilities to perform sustained work on demand. In this connection it is of interest that one of the generally accepted attributes of the aging human brain's function is its greater susceptibility to early fatigue during demanding tasks. Such rapid fatigueability would be expected to result indirectly from the loss of neurons' rDNA.

It has also been reported that rRNA synthesis decreases during aging of human cells in vitro (Bowman *et al.*, 1976). Perhaps also relevant is the observation (Macieira-Coelho, 1974) that ribosomal RNA synthesis is enhanced when the lifespan of fibroblasts in culture is increased through corticoid addition to the medium in which they are growing.

We and others have considered the possibility that there may be a sufficiently large ex-

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cess of rDNA at birth that even a 70% loss would be without measurable effect on an aging animal's function. This hypothesis seems unlikely to be correct for the following reasons. First, natural selection usually reduces the size or amount of components to those commensurate with efficient function during early life, because every excess of machinery in a system is derived from materials and energy expenditures that could be used by other systems. Weismann referred to this process as pangenesis—the loss through lack of selection pressure of parts that serve no specific function (Weismann, 1891). Second, in some systems, such as xenopus, a special amplification of rDNA genes takes place so as to meet the synthetic demands of early embryogeny. When these early demands are over, the extra rDNA (present as circular extra-chromosomal DNA) is destroyed. Third, in the case of the bobbed mutants of Drosophila, these genes' deficiency only has a visible effect during critical stages of development (specifically the period during which the bristles are growing at maximum rate prior to eclosion.) The life spans, however, of such mutants are reduced compared to those of their nonmutant counterparts. Previously unpublished experiments conducted in our laboratory indicate that this decrease in the life span of bobbed mutants parallels the severity of the bobbed phenotype (see Fig. 14). The decrease in lifespan was, however, only about 25% in these studies in the most extreme bobbed mutant studied. Similar or greater reductions in lifespan occur in many other Drosophila mutants (Pearl *et al.*, 1923) and it is not valid to contend that each of them represents premature aging.

David Doggett has studied the rRNA synthesis capacities of isolated T cell nuclei obtained from humans of various ages (Doggett, 1983; Doggett *et al.*, 1986). He discovered a decrease in the amount of rRNA synthesized/nucleus (in response to a prior challenge with a mitogen) by nuclei isolated from lymphocytes of humans of various ages. This decrease, however, appeared to be insignificantly small when the radioactivity incorporated into rRNA was "corrected" for the fraction of the total cells that responded to added mitogen. It can, of course, be argued that using data that represents the components of only the most active cells biases the interpretation of experiments against the detection of any significant age-dependent decrement in such functions. This would apply particularly if cells are unable

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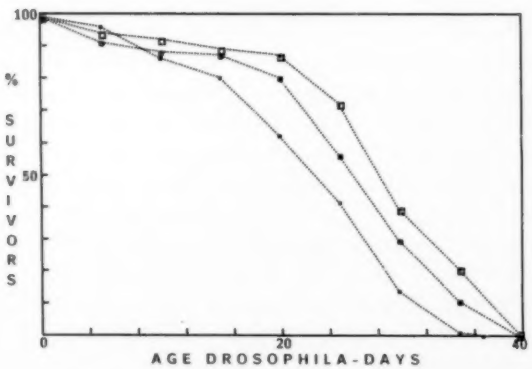

FIG. 14. Representation based on the effect of bobbedness on the survivorship of mutants and wild type Drosophila. The large squares depict the percent survivorship of "wild" type flies. The small boxes show the survivorship of mutant flies exhibiting a middle-range bobbed character. The lowest curve depicts the survivorship of extreme bobbed phenotype mutants. The mean age at death of the three groups are: 28.5, 25.9 and 23.1 days.

to respond to mitogen at all if their rDNA reserves are below a certain critical value. In other words, if the only cells responding to mitogen are precisely those that have sustained little damage, then one obviously would expect to find essentially identical rRNA synthesis capacities in such "hardy" nuclei selected from the pool of lymphocytes of old animals and the total responding lymphocyte pool present in young humans.

To conclude this section of the discussion, it is essentially certain that rDNA copies (as well, it seems likely, as other kinds of tandemly duplicated regions of DNA) are lost during aging in certain kinds of aging cells, particularly those present in postmitotic tissues. The magnitude of this loss (averaged over a population of cells) is such that some cells will statistically have lost nearly all of their original rDNA. Such cells will be greatly impaired in all of those functions which depend on the continual replenishment of rRNA – namely all protein synthetic activities. For the above reasons it seems most likely that age-dependent depletion of rDNA is the main origin of those gradual decrements in function that are the hallmark of human aging.

On the Mechanisms of Tandemly Duplicated DNA Loss

The most likely means through which rDNA loss occurs is analogous to the excision of episomes in prokaryotes and to the DNA rearrangements that occur in specific immune cell clones – rearrangements that provide the needed heterogeneity of potential responses to challenging antigens or aberrent cell surfaces. Of the other possible mechanisms suggested elsewhere (Strehler, 1978) the most likely is a transcription-dependent destabilization of tandemly duplicated genes or the less likely "slippage" model, i.e. slippage of sense and non-sense copies over each other so as to cause proper pairing but also to cause unpaired loops of single stranded DNA to be extruded and subsequently to be deleted as part of a "mis-repair" process. Discovering what the mechanism of loss is is important in determining what kind of measures might be taken to decrease the rate of this process (see Figs. 2, 4 and 5, pp. 456–460 of Strehler, 1978 for details).

Causes of Gene Excision

The most likely means (the episome-like excision model) through which tandemly duplicated gene loss occurs involves reactions quite similar to those that occur during meiotic crossing-over. It is also related to sister chromatid exchange observed in mitotic cells (see Tartof, 1973; Tice *et al.*, 1975). Although the precise mechanism through which crossing-over occurs is not fully documented, there seems to be general agreement that an initiating event is a single strand break in DNA. Such breaks are known to occur through the action of mutagens, but they also occur spontaneously and indeed are essential if normal meiotic crossing-over is to occur. Spontaneous single stranded breaks usually involve the hydrolysis of a phosphate to deoxy-ribose linkage in DNA. Such breaks occur under alkaline conditions in the test tube during ordinary hydrolysis of DNA for chemcial analytical study. It can be expected that a certain number of them will occur spontaneously even under neutral conditions in vivo if the proper amount of energy is released near to a given phosphate ester bond. Such energy could be supplied by local statistical heat fluctuations but would be accelerated by any reaction that released a large amount of energy in the vicinity of a DNA phosphate ester bond. Energetic reactions such as peroxide dismutation to water and oxygen and the reaction of free radicals (or alternatively the dissipation of the kinetic energy released by a high energy photon) could well provide the necessary activation

energy for hydrolysis. Alternatively, free radicals could produce their effects through attack on the carbon-hydrogen bonds in the deoxyribose components of DNA. The removal of an electron by an OH radical from the ribose skeleton would labilize the carbon to carbon bonds in that skeleton and produce a break in the carbon chain. Whether such carbon to carbon breaks occur (rather than phosphate ester cleavages) has not been determined experimentally to this writer's knowledge. It is also likely that single strand breaks are produced by specific enzymes at the time that meiotic crossing over occurs, so as to increase the probability that this evolutionary adaptive process (crossing-over) will occur at the proper time during gamete formation.

Once a single strand break occurs crossing over is believed to occur if there is a "peeling back" of the broken strand and the synthesis of an extension of the 3′ OH end of the broken strand on the template of the opposite strand. The peeled back segment is then available for pairing with a peeled back portion of the opposite strand in an adjacent complementary and nicked DNA molecule. The pairing of these complements establishes the initial basis for exchange between the DNA molecules. Subsequent steps are believed to involve the exchange of the other two strands followed by the necessary tying together (ligation) of these strands plus the synthesis or degradation of the number of nucleotide sequences needed to permit perfect exchange and thereby to yield two different repaired double stranded DNA molecules each made up of parts derived from two different DNA molecules.

It is not known whether the enzymatic apparatus needed for a meiosis-like chromosome exchanges is present in nondividing cells. The discovery that a small amount of DNA synthesis occurs even in such nondividing cells as human neurons implies that the needed machinery is present, even though perhaps in reduced amounts compared to a cell in meiosis. As noted earlier, it is known that the enzymatic apparatus that detects single stranded breaks is present in somatic cells and that this adenosine-diphospho-ribose transferring system "labels" the proteins surrounding the single stranded break, presumably so as to activate the needed repair enzymes or processes.

The sequence of events leading to the kind of exchange between separate DNA sequences required in order to excise an episome or to remove a segment of rDNA probably involves similar steps, as follows: (1) Nicking of DNA single strands; (2) synthesis along the 3′ OH end of the break according to the template provided by the other unnicked strand; (3) bringing the matching copy of DNA present at the same location in a different copy of the gene into apposition with the nicked strand; (4) nicking this apposed segment of DNA; (5) annealing the complementary, peeled back, segments of the two different DNA regions to each other; (6) completing the remainder of the crossover events which ultimately result in the formation of a nicked or closed DNA circle (subsequently degraded) plus a reduced length segment of the DNA remaining on the chromosome.

MODIFYING THE RATE OF DAMAGE

Single-Stranded Break Repair

If metabolically produced free radicals (for example those derived from the peroxide produced by the oxidation of reduced FAD or by xanthine oxidase) are the major endogenous nicking agent in mammalian cells then any evolved mechanism that reduces their concentration within cells would be expected to reduce the rate of rDNA gene depletion. It is interesting that such substances as uric acid are good free radical trapping agents (Ames,

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1981) and it may be that uric acid excretion as one means to excrete excess nitrogen has been retained among mammals because of the protection it provides against free radicals. It might be suggested that such animals as the dalmatian coach hound (which excretes large amounts of uric acid) would have longer lifespans than do dogs not so endowed. However, there does not seem to be an increased lifespan of such dogs although they may well die by virtue of other limiting genetic endowments.

A second means to reduce the rate of loss of rDNA gene copies would be to increase the efficiency of repair of single stranded nicks because the probability of excision crossovers should be a direct function of the number of unrepaired single-stranded breaks that exist within the rDNA genes. Thus, the rate of loss of these genes may differ between dogs and humans because of the number of such breaks that exist at steady state is greater in dogs than in humans rather than the number of breaks that occurs per unit time is greater in dogs than in humans.

There is no obvious way for an enzyme to "know" a priori whether a nick is an invitation to begin meiotic crossing over or the result of accidental molecular damage to DNA that should be repaired. But, if repair of a nick occurs before the events leading to crossing-over can be initiated, then that nick will not lead to DNA excision according to the mechanisms discussed here. Thus, the evolution of a system that either has a reduced rate of crossover initiation at the sites of breaks or one that initiates the repair of nicks more rapidly after they occur should also have a slower rate of aging due to loss of tandemly duplicated genes—a longevity-increasing evolved quality.

Replacement of Deleted Gene Copies

If, as is proposed here, the loss of tandemly duplicated genes through an abortive episome excision-like mechanism is the dominant uncompensated cause of aging among humans, a key question is whether such a process might be reversed in presently living or future generations of humans. The loss of different amounts of the same genetic material in different cells poses a very difficult repair problem for any enterprising genetic engineer. One can conceive of a viral vector that could carry the sequences needed to restore the lost genes to the proper dosage in each chromosome of each cell—but such a viral carrier of this information would have to be a very "smart" virus at the very least. It would have to be able selectively to add gene copies only to those locations from which they were lost earlier. This is a demanding task because it requires that an approximate measure of the number of gene copies present in a given DNA segment be made and that a decision to supplement the existing dose or not be made by such a system.

For the time being the prospect that such a vector will be bio-engineered in the near future seems dim. On the other hand if it should turn out that the decrease in the number of NOR's observed is due to the complete loss of the sequence of rDNA genes on a given chromosome, some kind of engineered repair might well be more feasible in the relatively near future because the detection of the complete absence of a long tandemly repeated segment of genes is a much simpler task than is the measurement of how many are left. The addition of a new set of such genes is also a much simpler task than is the insertion of precisely the proper number of copies required to restore some original number of such genes.

It is difficult to make any prediction regarding the real feasibility of such "molecular therapy" until more is known about the precise mechanisms involved in losses and until more experience has been acquired in the insertion of replacements for defective structural

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genes into cells in vitro and in vivo. A naive optimism that such abilities are just around the corner is only slightly worse than absolute pessimism that such is impossible—particularly in light of the truly remarkable advances in molecular biology and genetic engineering that have occurred during the last 15 years.

RESEARCH IMPLICATIONS

Even before (and if) it becomes firmly established that the limiting and dominating factor in human aging is the loss of such tandemly duplicated gene sequences as rDNA and possibly of other multiple-copy genes that specify tRNA's and histones, certain implications already exist regarding the most effective way to invest research funds in present and future aging research. Among these is an imperative to concentrate a certain amount of "risk capital" (e.g. 10–15% of total funds available) in exploring reasonable ideas that are testable regardless of how greatly they differ from current fads or areas of concentration. Conservatism with regard to the use of limited research funds is understandable and to some degree justified because the most likely payoffs in the short run are always extensions, however miniscule, of what is already known. In any event the "peer" system of review virtually insures that most highly imaginative new ideas will not be supported. Much readily supported research, which emphasizes the "development" component in research and development, is really only an exploitation of what is known with reasonable certainty. Most would probably agree that the really large advances in science and technology did not follow the Edisonian model, but rather were the products of individual scientific entrepreneurs who followed some seemingly impossible vision. One example in biomedicine is the discovery of antibiotics; another is the identification of DNA as the genetic material, its structure and the unravelling of the genetic code; a third is the bizarre concept that one could manufacture human polypeptide hormones, etc. in bacteria—a fantasy that was realized in record time because of basic studies on the nature of bacterial episomes and plasmids; or, fourth, that round worms could reveal something important about changes in the configurations of proteins that had applicability to human aging (Gershon and Gershon, 1970).

There has been a seeming overemphasis on the cliche word "aging model" and this emphasis has led to the funding of some studies that are not centered on the main ultimate object of aging research, the human animal. This is defensible on the basis that certain kinds of experiments cannot be performed on humans and animals are the only reasonable substitute. The "models" chosen are usually the least expensive ones to culture and maintain (fruit flies, Campanularia, vinegar eels and laboratory rodents), but they have life spans and biological properties that are almost certainly vastly different from those of humans. For the above reasons the results obtained with model systems are suspect in that they have no direct implications regarding the aging of humans unless and until discoveries on the models are extended to human materials. In short, one may derive a full understanding of how some short lived creature ages without shedding any light on what limits the life spans of men—because the "problems" that limit the life spans of short lived species have already been "solved" evolutionarily by long-lived species. So whenever feasible, human cells and tissues (or materials derived from similarly long-lived species) should be used if the object is the understanding of human aging.

The continued pursuit of such subjects as the loss of ability to divide after a certain number of doublings has transpired (clonal cellular aging) is valuable because such studies

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(see Hayflick, 1965) may supply insights into how the capacity of cells to divide is controlled during normal development, not because the aging of key human tissues necessarily occurs through similar mechanisms. Likewise, further studies on the accumulation of single strand scissions during aging (enlarging on the pioneering, but unconfirmed, discovery by Price and Makinodan) could most probably provide an understanding of events leading to such damage. The study of the means through which free radicals produce damage to DNA and other cellular components such as membrane lipids should provide a basis upon which to evaluate the means through which various intrinsic and environmental factors cause damage to cell constituents; fundamental studies of the role of ADPR transferase and the means through which depyridinated NAD is added to damaged chromosomal sites should generate insights into the events transpiring after single stranded DNA breaks occur in eukaryotes; finally, a complete understanding of the molecular mechanisms underlying the process of exchange between homologous DNA sequences and means to augment or reduce the frequency of such exchanges should provide both insight and perhaps means to modify the side effects of such molecular events as well.

There will, no doubt always, continue to appear new kinds of money-making fads, prescriptions for extended lifespan through esoteric diets or exercises, promises of new "breakthroughs" and books written by false prophets or wishful thinkers similar to those that have previously beclouded and still substitute hopeful science fiction for a truly scientific evaluation of the prospects for extension of human life. Like the flies that collect where the aroma of honey exists, these messengers of hope to those addicted to needs for self-prolongation will extract some tainted measure of material reward, but the promises will no doubt continue to leave the witless citizen in a sadder but wiser state.

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There is, of course, no reason to believe that there is anything magical about the three score and 10 years average longevity allotted to our primate species. The evolution of means to avoid or repair the damage that now limits lifespan certainly exists, but will require a kind of patience and selection of the fittest (longevity wise) that is poorly suited to the impatience of modern humans or the social reverence accorded by some to any form of life, including the lowest worms, cancer cells and hopelessly defective human embryos. If the evolution of further longevity is to occur this will require both a knowledge of what needs to be selected for (based on research findings) and a socially acceptable means for selectively propagating such genes in the descendents of present humans. Because of the otherwise admirable and understandable taboos of compassionate human societies it may be too late for such societies as ours even to try such experiments in "natural" bioengineering.

A second, more "humane" alternative is to invent a genetically engineered means to patch up whatever fails in individuals or to introduce, into the species' DNA, chemically synthesized genes that produce the new or superior products needed to compensate for current or newly emerging kinds of life-span limiting age changes.

A final, probably the most easily attained, alternative is to discover what processes (and structures) generated a conscious human entity: how self awareness and a joyful sense of life are generated within the machine we call a brain. When that is understood it will almost certainly become possible to mimick a human brain in some other (perhaps silicon-chip) form. The tools to achieve this last means for extending individual human consciousness appear to lie just around the scientific-technological corner. Whether such a surrogate self is preferable to no self at all can be decided by each aging biological self at an appropriate time. Hopefully, such surrogates will possess ample quantities of the best of human

qualities including an appreciation of Michaelangelo's metaphoric prescription's applicability to scientific adventures and social value codes, and particularly how what is done today and tomorrow will affect the lives of humans the day after tomorrow.

Dedication—This paper is dedicated to my mentor, colleague and friend of 30 years, Nathan W. Shock, and to the memory of my daughter, Carol Theodora (1953–1956).

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AGING AS ENTROPY

WALTER M. BORTZ II

Palo Alto Medical Foundation, 300 Homer Avenue, Palo Alto, California, 94301

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> At the present time it is impossible to tell whether all the pieces of the aging puzzle are at hand—until a general theory appears that will unify the facts—no one can offer more than a informed guess as to where the key facts lie. (Moment, 1982)

WHY DO aging and disuse look alike? This little question, like the simple aphorism "Use it or lose it" appears trite and trivial. Yet, understanding of this homology, I propose, holds profound significance.

With such variety of structural and functional changes to choose from why should disuse select aging as a model to mimic? Do aging and inactivity contain some shared element which is similarly expressed in each, or do the phenomena interact? In an effort to provide insights it is important to search out common terms—both aging and disuse involve the effects of energy flow on matter over time. Answers must transcend biology inasmuch as all substances age, stars, Chevrolets, redwoods, tadpoles. Changes due to inactivity likewise extend to inanimate circumstances, stream beds, iron pipes, city slums.

In light of this universality it is logical to look to unifying canon. Szent-Gyorgy (1957) recognized this as he wrote "Biological phenomena, possibly, are to a great extent the expression of subtle changes which take place in dimensions unknown" which "belong to the realm of quantum mechanics and can be described only in its language."

We find the terms described in the Second Law of Thermodynamics which interrelates energy and matter over time. It is a sort of cosmic Murphy's Law. It states that an isolated system left to itself will, in the course of time, go toward greater disorder. The statistical tendency of matter to go into disorder is unidirectional and inviolable. Every particle of the universe participates in this process. No matter is static. At the submicroscopic level matter is in violent agitation, at the macroscopic level flux is constant (Morowitz, 1968).

Within a system certain parts of the system may organize into a state of greater order as in the formation of a crystal from a solution, but the system as a whole exhibits increased disorder (Blum, 1968). The disorder is called entropy. Entropy is quantitated in units, cal-

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ories per degree. Entropy is a measure of the amount of energy that is no longer capable of conversion into work and thereby is the force which drives chemical reactions.

The second Law is formulated

$$\Delta G = \Delta H - T\Delta S$$

where G is free energy, H is heat, T is the absolute temperature at which the reaction is occuring and S is entropy.

Thermal physicists have looked at physical events to gain insight into theoretical biology. The classic realm of the thermal physicist is the closed, equilibrium system in which reversibility is an inherent concept. Our biosphere, however, is open in which nonequilibrium is the rule. Thereby additional hypotheses are implied. Chemical reactions are prototypical nonequilibrium, nonreversible process. Prigogine, Morowitz, Dyson, and others provide us with a new thermodynamic paradigm of immense importance. Nonequilibrium brings "order out of chaos" (Prigogine, Stengers 1984). "On the dry bones of the nature of atoms and the distribution of energy in the universe are assembled the flesh and blood of life" (Prigogine and Stengers, 1984). Biologic phenomena are ultimately the consequences of laws of physics.

Energy flux has led the physicist to hypotheses that bear on the nature of life. "In the steady state system the flow of energy through the system from a source to a sink will lead to at least one cycle in the system" (Morowitz, 1968). This is known as the Cycling Theorem. It means that molecular organization and cycles occur in any system undergoing energy flow, and are therefore not confined to living processes. Cycling occurs as a result of the flow of compounds of high chemical potential to compounds of less chemical potential, some of which leave the system. Rhythmicity is a general phenomenon. The primary cycle of our system is generation of glucose by plants, its combustion to CO_2 and water, and subsequent resynthesis.

Continuous work leads to self-organization, and the ordering of the biosphere. "Life is the supreme expression of self-organization processes" (Prigogine and Stengers, 1984). Much organismic work is devoted to the maintenance of the constancy of the internal environment. This phenomenon is remarkable throughout nature considering the diversity of temperatures and other environmental stresses which confront life. Entropy, which Eddington (1958) termed "time's arrow" in giving direction, is ordering. The biosphere is represented as "a kind of steady state between the buildup of ordered macromolecular structures using photosynthetically acquired energy, and the breakdown of these structures owing to random thermal noise" (Prigogine and Stengers, 1984).

Life is the bundling and ordering of energy. Metabolism is the language of life. Its syntax is the complex set of competing synthetic and degradative biochemical reactions. Its meter is the oscillation of enzymatic processes and allosteric feedback control loops. The major statement of metabolism concerns the pulling off of electrons and protons from a reduced carbon compound, and their subsequent use as energy to run a living cell.

In this development new order is apparent as a seeming contradiction to the Second Law and its entropic compulsion. Schrodinger (1947) wrote "To reconcile the high durability of hereditary substance (DNA) with its minute size, we had to evade the tendency to disorder by 'inventing the molecule,' in fact, an unusually large molecule which has to be a masterpiece of highly differentiated order, safeguarded by the conjuring rod of quantum theory."

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A molecule is a "negentropic" device. On a more macroscopic level enzymes are proteins which lower energy barriers between reactants and products—they are biologic catalysts.

A plant or animal is a complex, highly organized and improbable event. They have low entropy. They are unstable, open thermodynamic systems which extract free energy from the sun and food respectively, and render back low energy, high entropy products of CO_2 and heat. Schrodinger (1947) wrote "All life survives by continually drawing from its environment negative entropy. What an organism feeds upon is negative entropy—it continues to suck orderliness from its environment." Morowitz (1968) wrote "In order for a system to prevent drift to equilibrium it is constantly necessary to perform work to move the system back into the improbable state that it is drifting out of." Atkins (1984) wrote "Coherence is intrinsically transient, and crumbles into incoherence when the structure ceases to be driven by a flow of energy."

Our biosphere is continually being degraded and reconstituted out of the same mass of chemical constituents (Morowitz, 1970). The flow of energy is accompanied by the formation and hydrolysis of phosphate bonds, usually those of ATP. ATP is the principal energy storage compound and is the "gear wheel" for our biochemical engine (Atkins, 1984). It is the major chemical link between energy yielding and energy requiring cellular activities. It is used to do biosynthetic, osmotic, and mechanical work.

ENERGY FLOW AND AGING

Hansford (1983) has recently reviewed the bioenergetics of aging. He began "Aging animals tend to be less energetic. It is therefore understandable that gerontologists have long focused attention on energy-transducing pathways in their search for the biochemical lesions which underlie the observable physiological decrements of old age." Innumerable studies have been performed both on intact tissues and fractionated cell preparations of mitochondria and cell sap.

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Hansford (1983) surveyed predominantly the impact of aging on the oxidative pathways. Most studies indicate that the oxidation of a number of substances is impaired with aging. This seems to be in accord with the observation that basal oxygen requirements decline with age. However, lean body mass also declines with age, so that the decline in oxygen requirement may simply be a reflection of less tissue. Additionally, Goldstein *et al.* (1982) have found the ATP concentration and oxygen consumption to be unaltered in tissue culture nests of aging human fibroblasts. Balmer (1982), however, reported a decrease in the body energy content of aging fish.

VO_2max, which reflects maximal oxygen consumption under exercise, high load, stimulation does decline with age. Therefore measurements which are static and not dynamic may yield artifactual inference. It is important to note that some observed oxidative defects are reversible by an exercise program. The interraction of aging and disuse is affirmed.

It is uncertain where the primary locus (loci) for any metabolic decrease with aging is seated. Prominent mention is made of membrane change. Alterations which result in decreased fluidity and permeability may be central. Ion transport and carrier protein-lipid interactions may have profound metabolic effects. The work of Harman (1980) has displayed the metabolic havoc which results from the cross-linkage of membranes. Peroxidation attacks the unsaturated fatty acids of membranes making them more rigid

(Leibovitz and Siegel 1980; Nohl, Brenninger and Hegner, 1978). Membrane lipid peroxidation and decreased mitochondrial function is also seen in aging plants (Munkres 1979). Accumulation of peroxidation products in the mitochondria gives rise to decreased ATP production, and thereby a decreased ability to maintain cellular order and homeostasis (Boveris, Oshino and Chance, 1972; Chance, Sies and Boveris, 1979). Superoxides formed as the natural result of metabolic processes lead to the depurination of nucleic acids and the recemization and deamination of proteins.

Irradiation has been used as an experimental tool to induce "age changes." This disordering influence leads to increased intracellular noise and disruption of vital processes. Conversely, hypothermia has been identified as one technique which retards aging. On theoretical grounds Dyson (1979) has indicated that hibernation represents a potential relief from age changes. Entropy is retarded by lowering of temperature. It is zero at $-273°C$.

Metabolic processes and homeostatic integrity depend on the capacity of the organism to free itself from the entropy which it cannot help producing. Rubner (1908) proposed the "rate of living" theory of aging. He observed, as have Pearl (1928) and others (Strehler and Mildvan, 1960; Cutler, 1980) since, that across a wide spread of species longevity is inversely proportional to the rate of energy transformations and cellular biochemical processes, i.e. basal metabolic rate. In general, life span increases with decreased BMR or increased body mass. This relationship can be artificially manipulated in the house fly (Sohal and Donato, 1978). As BMR is increased by heating and/or increased flight activity life span is curtailed from 50 to 25 days. The accumulation of fluorescein age pigment correlated directly to BMR variations. Economos (1981) refined the rate of living theory by noting that countertropic mechanisms such as scavenging of free radicals and antioxidations, and proteolytic enzymes which selectively remove abnormal proteins are likely to be differentially present in various species, and can therefore account for the variability noted in Rubner's general formulation. Similarly, Sacher (1967) observed "Aging is not the result of metabolic activity per se but rather of the production of entropy concomitant with metabolic activity. Aging can no longer be considered as simply a question of how much metabolic work; it is also a function of how well the work is done in thermodynamic and informational terms."

One of the key experiments in aging research was conducted in 1935 by McCay (1935) at Cornell. He observed that life span of laboratory rats could be substantially extended by underfeeding them and stunting their growth. Growth rate and longevity seemed to relate. This is likewise seen in drosophilia—a metabolic clock appears to be working. Buffon noted 200 years ago that life span seems reciprocally related to time of sexual maturity. Masoro (1984) relates the age slowing effect of food restriction to the retardation of "age-related physiologic and immunologic deterioration." Walford (1983) and others (Schneider, 1980) have described the decreased capacity of older animals to repair DNA as a central feature of aging. Despite similar BMR's humans live twice as long as apes; cell cultures of human cells demonstrate twice the DNA repair capacity of apes (Hart & Setlow, 1974). It is challenging to try to conform these central observations to general thermodynamic theory.

AGING, HOMEOSTASIS AND ENERGY FLOW

Sacher (1978) and others (Rowe and Minaker, 1985) ascribe aging to a decline in homeostatic capacity over a wide range of function. "The hallmark of aging is a progressive de-

crease in homeostatic capability—a decreased response to an internal or external environmental perturbation" (Leaf, 1984). I am confident that the physicists would describe homeostatic mechanisms as macrostatements of the ensemble effects tending to order the system. Homeostasis is reciprocal to aging as energy production retards entropy.

ENERGY/ENTROPY ≅ HOMEOSTASIS/AGING

I have been struck forcibly by the coincidence of biologic age changes to those of disuse (Bortz, 1982). A cataloguing of age changes in geriatrics textbooks is a virtual mirror image of changes seen as the result of the disuse of physical inactivity. This coincidence applies at all levels of subcellular and cellular organization and is generalized throughout nature. It cannot be coincidental that a casted leg has every characteristic of a leg forty years older than the other. As energy is applied to any part of the body it thrives—when energy throughput is denied to any part it withers. This truth has led to the formulation of "The Disuse Syndrome." Its features are (1) cardiovascular vulnerability, (2) musculoskeletal fragility, (3) obesity, (4) depression and (5) precocious aging—all common experimentally produceable, and predictable outcomes of inactivity (Bortz, 1984). Lakatta (1983) has shown that the changes in many cardiovascular parameters which had classically been ascribed to aging are reversible by an exercise program. Even more compelling is the observation of Dustman *et al.* (1984) that an exercise protocol used in a group of older persons not only improved their neuromotor facility but improved their cognitive functioning. Disuse is accelerated aging. It is striking to observe that the decline in functional capacity of most body systems occurs at approximately the same rate, 1% per year. This would seem to indicate that a universal principle is applied to the entire organism (Cutler, 1975).

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As disuse has many-fold impacts on aging processes, Selye (1936) remarked that stress, too much energetic throughput, leads to changes which bear a striking resemblance to those of aging. Granting this similarity as well, the model emerges that energy throughput when nonoptimal either by its insufficiency or excess leads to premature deterioration of the organism. Stated positively, vitality of the organism depends on the appropriate energetic stimulus. This truism seems valid when viewed at the subcellular, tissue, and total organism levels. Numerous examples of servomechanisms exist. Synthetic and oxidative enzyme levels are highly sensitive to the level of the product and precursor respectively. If there is a surfeit of the product of a synthetic enzyme system, levels of the enzyme decline. Conversely, if there is an abundant substrate available for degradation, oxidative enzyme levels rise, whereas as combustion substrate is depleted the enzyme declines.

At the tissue level, if insufficient load is applied to muscle or bone, atrophy and osteoporosis result. When too much is applied, rupture and fracture ensue. When the cardiovascular system is not challenged, an array of catabolic changes result leading to decreased performance indices. When the cardiovascular system is overworked, failure results. When the CNS is stimulated new dendritic branching ensues, when insufficiently challenged decreased performance appears as delayed neuromuscular and cognitive responsiveness. Sensory deprivation leads to disorientation and psychotic depression. Excessive throughput, stress, leads to hyperkinesis and unreality (amphetamine psychosis).

At the whole organism level insufficient energy flow breeds apathy, obesity and multiple vulnerabilities. Excessive throughput leads to what Selye characterizes as the general adaptation syndrome—nephrosclerosis, ulcers and hypertension.

The cybernetic model of modulated energy flow with its attendant ordering fluctuations appears to conform to the cycling theorem proposal of the thermophysicists.

The rate of living theory indicates that within certain broad bounds life span and intensity of energy throughput are inversely related—the faster it goes, the sooner it wears out. This is in accord with the Second Law. It is fascinating to observe, however, that it is this same energy throughput which maintains vigor and order throughout life, in consonance with the recently elaborated theories concerning nonequilibrium thermodynamics. Entropy drives a directed life course, and simultaneously provides a source of ordering of that life course. There is accumulating evidence in man and animals that a life actively lived not only extends that life, but lends vigor and capacity to its content.

Aging is a restatement of "time's arrow." It is inexorable, inescapable, and ultimate, but it is no longer so inscrutable thanks to the conceptual base provided by the thermophysicists. This conceptual base of aging should aid in directing and consolidating clinical strategies.

When aging is viewed as a thermophysical event, it is implicit that its origin is intrinsic to the organism. It is the result of molecular decay inherent in the finite rate of entropy production in metabolizing systems. In such a conception death becomes a moment of disruption of ordering capacities, hence a rapid step up in entropic decay and a rush toward equilibrium. The standard definition of death concludes that life ends when the heart stops. This says, in effect, that oxygen supply is the final arbiter of living processes. Life goes on when reproductive, digestive, excretory, intellectual function is lost, but cardiorespiratory arrest, or cellular poison with a respiratory toxin, cyanide, leads to near instant death. Oxidative metabolism is our most primitive function which cannot fail if the energetics of the organism are to be maintained. Strehler and Midvan (1960) comment "An organism lives or dies according to whether its maximum power output in the challenged modality is sufficient to overcome the disruptive influence of the challenge." When energy flow is interrupted, all ordering effects are lose, entropy prevails. Death, like entropy, is intractable.

Finally, Prigogine (1984) wrote "Energy flow causes fluctuations which may be too great for the system to absorb. This leads to reorganization and thus greater complexity and the greater likelihood of random fluctuation which leads in turn to still more instability and reorganization. The sum total is the quickened creation of living matter through successive diversification into new structures, i.e. evolution." Lotka (1922) argued that "natural selection favors those organisms that are able to increase the total mass of the system, the rate of circulation of mass through the system, and the total energy flux through the system so long as there is presented a residue of matter and untapped energy."

Our own evolutionary history has been that of an extremely active species (Bortz, 1985). The last few hundred years have redirected the vigor of our existences. As our energy components decrease, our order will decrease, our entropy will increase, we will age inappropriately. Stagnation, lethargy, and apathy breed molecular, cellular tissue, organismic, societal decay. As physicians we should look to this cosmic imperative and incorporate it daily into our clinical strategies.

SUMMARY

Like the weather, many people talk about aging, but no one seems able to do anything about it. This implacability derives, at least in part, from our inability to provide a conceptual base from which analytyic insight might derive.

The emerging field of nonlinear thermodynamics presents a new view of the universe. It relates matter and energy in the framework of time. Entropy, "time's arrow," gives direction. Energy, rendered coherent through the metabolism of biology, acts to retard dissipation as entropy.

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For physicians, particularly those interested in geriatrics, this provides the challenge to understand how these forces are inscribed onto the human condition. The homeostatic controls which order our existence are energy dependent. As energies diminish, homeostasis as order deteriorates, aging proceeds and life is threatened. It seems to follow that if we are to diminish the entropic declines of aging our clinical strategies should emphasize actions which might retard the disruption of ordering processes. Homeostasis is at risk to the ravages of disease. This has been the standard business of medicine. But homeostasis is manifestly determined by the ordering effects of an energy flow. Either too little energy flow as disuse, or too much, as stress, lends further major burden to homeostatic controls. Every body system, at every level of organization, is beholden to this mandate.

As our wisdom stretches to encompass the notion that aging and illness have other dimensions than just disease our view of ourselves will become more whole and in enlarging consonance with universal law.

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FACTORS AFFECTING ATTRITION IN THE BALTIMORE LONGITUDINAL STUDY OF AGING*

SUSHIL K. SHARMA

Department of Mental Hygiene, School of Hygiene and Public Health, Johns Hopkins University, 615 Wolfe Street, Baltimore, MD

JORDAN D. TOBIN

Applied Physiology Branch, Gerontology Research Center, Baltimore, MD

LARRY J. BRANT

Longitudinal Studies Branch, Gerontology Research Center, Baltimore, MD

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Abstract—In a longitudinal study of aging, the influence on attrition of age, marital status, education, occupation, distance between a subject's residence and study center, self-health and financial assessment, and method of recruitment was examined. Prospective follow-up information on the subsequent active/dropout and alive/dead status on the 1088 subjects, who joined the Baltimore Longitudinal Study of Aging during the first twenty years (1958–1977), were studied using a proportional hazards model. Three analyses, each controlling for death in a different manner, produced similar results. The analysis indicates that age, education and distance have the strongest association with attrition ($p < 0.001$), while self-health assessment has a lesser association ($p < 0.05$). The lowest risk in age, for example, occurs in 40–49.9 year olds with subjects less than 30 having an increased risk of 91% and subjects 70 or older having an increased risk of 189%. Examination of attrition probabilities show that subjects living 500 or more miles from study center, ages 70 and older, with less than bachelors degree, and perceiving their health as average or below have the greatest probability of dropping out—approximately 3-fold greater than the average subject's probability.

Key Words: risk factors, study retention, dropout, long-term studies, human, gerontology, research methods

INTRODUCTION

THE DESIRABILITY of longitudinal research to study the aging process is well documented in the literature (Birren, 1959; W.H.O., 1959; Sussman, 1964; Butler, 1967; Riley and Foner, 1968; Palmore, 1970; Andres, 1974). However, there are a number of identifiable

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Correspondence to: Dr. S.K. Sharma, Department of Mental Hygiene, School of Hygiene and Public Health, Johns Hopkins University, 615 N. Wolfe St., Baltimore, MD 21205.
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administrative and methodological problems inherent in the longitudinal method of research. These include the uncertainty of long-term funding to support the cost of such research, the need to obtain the required interest and commitment of participating scientists, and the special methodological requirements of longitudinal research (Jones, 1958; Botwnick, 1964; Shapiro *et al.*, 1969; Busse, 1970; Andres, 1974).

Longitudinal research on human subjects is dependent upon the availability of individuals at regular intervals for repeat examinations. Thus, to a large extent, the success of any longitudinal study depends upon the subjects to make an ongoing commitment to cooperate and remain in the study, since longitudinal analyses depend on repeat data on the same individuals as they age. However, despite the fervent desire to maintain 100% cooperation from the subjects, attrition over time is one of the major problems in longitudinal studies (Jones, 1958; Goldfarb, 1960; Rose *et al.*, 1976; Spear and Zold, 1971; Parnes, 1972; Andres, 1974). Attrition in longitudinal research can occur not only by reason of death, but by refusals due to diminution of motivation or interest, change in circumstance (e.g. health and economics), or a loss of contact resulting from change of residence. Different rates of attrition on longitudinal studies have been reported in the literature (Lowenthal and Boler, 1965; Riegel *et al.*, 1967; Granick and Patterson, 1971; Bell *et al.*, 1972; Powers and Bultena, 1972; Wilson and Webber, 1976).

A comparison of rates is difficult since each study represents a unique population. Each study has different methods of recruitment, from different populations, with different rates of initial participation, and different strategies for repeat visits and retention as well as different definition of drop-out. Every study, therefore, may be considered nonrepresentative of the general population and results must be generalized with caution. Agreement among the studies, as with epidemiological studies in general, lends credence to the results.

Because longitudinal studies require the same individuals to return at regular intervals for repeat examinations, findings from only those longitudinal studies that have reported some information on drop-outs will be reviewed here. Factors that have previously been examined can be classified as (1) demographic characteristics, (2) psychological attributes, and (3) physical health measures. It must be pointed out that even in these longitudinal studies many of the conclusions made about drop-out subjects are not clear cut. In this paper, only findings pertaining to demographic variables are reviewed. The major findings were that, in general, subjects who drop out are likely to be: (1) older (Riegel *et al.*, 1967; Atchley, 1969; Powers and Bultena, 1972; Wilson and Webber, 1976); (2) not married (Maddox, 1962); (3) less educated (Berelson *et al.*, 1954; Rose and Bell, 1965; Streib, 1966; Wilson and Webber, 1976); (4) in stressful or high demand occupations (Streib, 1966; Rose *et al.*, 1976); (5) financially less well off (Streib, 1966; Atchley, 1969); and (6) in poor health (Maddox, 1962; Streib, 1966; Atchley, 1969; Powers and Bultena, 1972; Wilson and Webber, 1976), as compared to subjects who remain active in the study.

With regard to age, Rusin and Siegler (1975) point out that the reasons for the finding that the average dropout is older than the average participant is the high number of deaths among older subjects. Although not clearly stated, they pointed out an important methodological distinction between (1) drop out because of death, and (2) voluntary drop out. In essence, they recognized death as a competing risk with dropping out, warranting special attention in the analysis.

The analysis presented here addresses this problem by comparing results using data of all deceased subjects; ignoring data of all deceased subjects; and using data of only those

deceased subjects who died while still considered active participants in the study. In addition to those study variables just mentioned, we have also examined the effect on attrition of (1) distance between subject's residence and the study center and (2) method of recruitment.

The purpose of this study is to examine the effect of several identifiable characteristics of subjects at first visit on subsequent attrition. It is beyond the scope of this paper to discuss the mathematical theory of how attrition effects the analyses of longitudinal data. Knowledge about these factors which lead to high or low attrition for reasons other than death may allow one to adopt strategies aimed at minimizing the attrition of subjects in this and similar studies.

MATERIALS AND METHODS

The Baltimore Longitudinal Study of Aging (BLSA) was initiated in 1958 to study the physiological, biochemical, sociological and psychological changes that occur with advancing age (Shock *et al.*, 1984). The recruitment of study subjects in BLSA is an ongoing one, and is entirely on a volunteer basis. There are three major modes of subject recruitment. First, most subjects are recruited by relatives and friends who are already subjects in the study. Second, some subjects are self recruits who learned of this study by reading articles in local newspapers or professional journals. Lastly, a few subjects are recruited by the BLSA staff.

The subjects are expected to return to the study center (Gerontology Research Center) at regularly scheduled intervals for 2½ days of intensive physiological, biochemical, psychological, and sociological examinations and tests. From the inception of the BLSA until August 1973, subjects under 70 years of age were expected to return every 1½ years while subjects over 70 years of age were expected to return every year. In August 1973 these criteria were changed to the following: (1) men under 60 years of age were to return every two years; (2) men 60 and over but under 70 years of age were to return every 1½ years; and (3) men 70 years of age and over were to return every year. Subjects reside in different parts of the U.S. They visit on their own time and at their own expense and provide their own transportation. No medical care is provided. However, all subjects are expected to have a family physician to whom medical reports are sent.

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The study population used in the present study is limited to those 1088 subjects who joined the study between February 1958 (the inception of the study) and June 30, 1977 (Table 1). As of the latter date, 658 subjects were activeiy participating in the study, 299 subjects had dropped out from the BLSA (46 of these first dropped out but died later on) while 131 subjects died while on active status in the study. A dropout is defined as a person who formally notifies the BLSA staff in writing or verbally, in person or by phone, of his wish to withdraw himself from future participation in the study; or a subject who has not returned to BLSA within three years since his last visit and who has not responded to attempts made by the appointment clerk to reschedule him for the next visit.

The distribution of the total, active, and dropout groups with regard to the eight risk factors selected for this study is shown in Table 2. They were all males who ranged in age from 18 to 96 years at first visit (mean age 49 years). Most subjects were caucasian. Most were married (87%), highly educated (44% have Masters, Ph.D. or M.D. degrees). In general, they represented a rather healthy segment of the population.

In order to identify risk factors for dropping out from the BLSA, a proportional hazards regression analysis as introduced by Cox (1972) and discussed and generalized by

TABLE 1. DISTRIBUTION OF SUBJECTS BY STATUS IN THE STUDY AS OF JUNE 30, 1977.

Groups			
	Total Number of Subjects	1088	
	Total Number of Active Subjects	658	789 Total Active
A.	Number of subjects who died while on active status in the study	131	
			177 Total Death
B.	Number of subjects who first dropped out and then died at a later point.	46	
			299 Total Drop out
C.	Number of subjects who dropped out and were known to be alive as of June 30, 1977.	253	

Kalbfleisch and Prentice (1980) was used. For a given subject the proportional hazards function may be written

$$h(t) = h_0(t) \exp(\beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_n X_n)$$

where $h_0(t)$ is the baseline hazard function with all the X variables set to zero; $X_1, \ldots, X_n$ are the independent or risk factors believed to affect attrition; and $\beta_1, \ldots, \beta_n$ are the regression coefficients or parameters to be estimated from the data. The independent variables or factors in the hazards model included: (1) age (2) marital status (3) education (4) occupation (5) distance between subject's residence and BLSA (6) self-health assessment (7) self-financial assessment and (8) method of recruitment. The proportional hazards model assumes that the log of adjusted hazard rate is a linear function of the covariates.

A maximum likelihood analysis was used to obtain estimates of the parameters in the hazards model where the likelihood function is based on the product of conditional probabilities of attrition of each time where a dropout was observed. This was accomplished using an expanded version of the FORTRAN program given by Kalbfleisch and Prentice (1980). The estimates of the parameters can be interpreted as the logarithms of the estimates of the relative risks of dropping out for each corresponding factor. In addition, the procedure used in this program estimates the baseline hazard function $h_0(t)$ using maximum likelihood estimation where no assumptions were made regarding $h_0(t)$, thus allowing it to take any shape (Cox, 1972).

Using these estimates of the parameters in the proportional hazard model, relative risks for each factor are obtained for each of its classes relative to a particular class. The hazards model with its corresponding estimates was then used to calculate survival and hence attrition probabilities as functions of follow-up (time) and the independent variables. Next, these estimated probabilities were used to calculate standardized attrition probabilities. These ratios measured the increased attrition probability of being in a particular class of a given factor, while the effects of all other specified factors were held constant. The estimated standardized attrition probability (SAP) for a given factor and class was

defined as the percentage of corresponding attrition probability to the overall average attrition probability. The overall estimated average attrition probability (OAAP) is the attrition probability obtained by weighting each factor's classes according to the study population distribution, thus providing a probability for overall average attrition.

RESULTS

First, a univariate analysis of the data independent of the lengths of follow-up on all 1088 subjects was performed. Age, education, and distance between a subject's residence and the study center were found to be significantly associated with attrition as indicated by a chi-square test (Table 2). The crude drop out rates indicated that (1) the risk of dropping out increased with increasing age, (2) increasing distance between subjects' place of residence and study center, and (3) subjects with less than B.A. degree had higher risk of dropping out.

Second, a proportional hazards function analysis was used to identify significant risk factors for dropping out from the BLSA. In this analysis, subjects in Group A ($N = 131$, Table 1), who died within three years of their last visit, were considered as active subjects in the program with a duration in study up to the time of death; and subjects in Group B and Group C ($N = 299$) were considered as dropouts.

Since subjects are obviously at risk of dying before having an opportunity to drop out of the study, death is, as a matter of fact, a competing outcome with dropping out. To control for the possible effect of death on dropping out, two separate additional hazard function analyses were performed. In a second analysis, subjects who died while on active status (Group A, $N = 131$) were excluded from the analysis, while in the third analysis, all deceased subjects (Group A and Group B, $N = 177$) were excluded from the analysis. All three analyses produce consistent results with age, education and distance between subject's residence and BLSA having the strongest association with attrition ($p < 0.001$). Self-health assessment ($p < 0.05$, $N = 1088$) was also found to be associated with attrition. In this section, we report in detail the results of the analysis with all 1088 subjects (analysis 1 only). The results of the other analyses will be discussed in the Discussion Section.

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Table 3 shows the corresponding estimates, standard errors for the parameter coefficients in the proportional hazards model, and the relative risks calculated using these coefficients for each of the eight risk factors. Table 3 takes into account person-years of follow-up and therefore the results are not directly comparable to Table 2 which does not. Table 3 also shows the standardized attrition probabilities (SAP) for each class of the eight risk factors (1088). The SAP represents an estimate of the percentage of OAAP (0.221) that we would expect if every subject in our study were in the same class of a given factor and the effect of the other factors were removed.

The proportional hazards model analysis revealed the following factors as demonstrating a statistically significant influence on attrition (Table 3, relative risks and standardized attrition probabilities).

1. *Age.* The relative risks for age at first visit were found to be significantly related to attrition ($p < 0.001$). The risk of dropping out increases by a multiple of 1.91 for subjects <30 years when compared with subjects between 40–49.9 years, while for those ≥ 70 years the risk increases by a multiple of 2.89. The standardized attrition probability (SAP) ranged from 68 for subjects who entered the study between the ages

TABLE 2. DISTRIBUTION OF ALL 1088 SUBJECTS IN THE BLSA AND CRUDE DROP OUT RATE FOR VARIOUS RISK FACTORS AT FIRST VISIT.

	Study Population			
	N	Percent	Crude Drop	
Factors	1088	100	Out Rate	χ^2
Age (yrs)				
<30	134	12	18	
30-39.9	215	20	24	
40-49.9	236	22	25	
50-59.9	188	17	30	
60-69.9	150	14	35	17.9
≥70	165	15	35	(p<0.005)
Marital status				
Never married	77	7	22	
Married	943	87	28	1.5
Others (S.W.D.)	68	6	31	(N.S.)
Education				
<BA	273	25	36	
BA	335	31	25	12.9
>BA	480	44	25	(p<0.005)
Occupation				
Professional	912	84	27	1.1
Non-professional	176	16	31	(N.S.)
Distance (miles)				
<50	670	62	25	
50-99	290	27	29	
100-199	48	4	38	
200-499	49	4	39	9.9
≥500	31	3	39	(p<0.05)
Self-health assessment				
Average or below	74	7	35	2.5
Above average	925	93	26	(N.S.)
Self-financial assessment				
Less than comfortable	178	18	31	
Comfortable	696	71	25	4.1
More than comfortable	105	11	32	(N.S.)
Method of recruitment				
Staff recruitment	32	3	34	
Self recruitment	123	11	28	0.79
Chain recruitment	933	86	27	(N.S.)

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40–49.9 to 169 for subjects who entered at age 70 or over. These values indicate a 32% reduction in the overall average attrition probability (OAAP = 0.221) for subjects ages 40–49.9 and a 69% increase in the OAPP for subjects 70 years of age or older.

2. *Education.* Relative risks for level of education at first visit were found to be significantly associated with dropping out ($p < 0.001$). The risk of dropping out for subjects with more than a bachelors degree is 0.47 that of the risk of subjects with less than a bachelors. The SAP ranged from 84 for subjects with more than a bachelors degree to 161 for subjects with less than a bachelors degree. These values indicate a 16% reduction in the OAAP for subjects with more than a bachelors degree and a 61% increase in the OAAP for subjects with less than a bachelors degree.
3. *Distance.* Distance between subject's residence and BLSA was found to be a significant factor ($p < 0.001$). The drop out risk of subjects living 500 or more miles away increases by a multiple of 2.36 as compared to the risk for those being under 50 miles from the GRC. The SAP increases with increasing distance from 89 for subjects living less than 50 miles away from GRC to 182 subjects living more than 500 miles away from GRC. These values indicate a 11% reduction in the OAAP for subjects living less than 50 miles away from GRC and a 82% increase in the OAAP for subjects living 500 miles or more away from GRC.
4. *Self-health assessment.* Self-health assessment was found to be a significant factor ($p < 0.05$). The relative risk for those with above average health was 0.65 that of the average or below group. For subjects who rated their health as above average the SAP

TABLE 3. ESTIMATES, STANDARD ERRORS, RELATIVE RISKS AND STANDARDIZED ATTRITION PROBABILITIES (SAP) FOR THE PARAMETERS IN THE PROPORTIONAL HAZARDS MODEL.

Factors	β	Standard Error	Relative Risk	SAP (Percent)[c,d]
Age (yrs)				
<30	0.648	0.277	1.91	120
30-39.9	0.263	0.210	1.30	86
40-49.9	-[a]	-	1.00***	68
50-59.9	0.331	0.207	1.39	91
60-69.9	0.612	0.209	1.84	117
≥70	1.060	0.212	2.89	169
Marital status				
Never Married	-[a]	-	1.00	84
Married	0.209	0.333	1.23	101
Others (S.W.D.)	0.267	0.421	1.31	106
Education				
<BA	-[a]	-	1.00***	161
BA	-0.722	0.178	0.49	87
>BA	-0.760	0.172	0.47	84
Occupation				
Professional	-0.328	0.209	1.00	104
Non-Professional	-[a]	-	0.72	78

Table 3 Cont.

Distance (miles)	0.213[b]	0.056		
<50			1.00***	89
50-99			1.24	108
100-199			1.54	129
200-499			1.91	154
≥500			2.36	182
Self-health assessment				
Average or below	-[a]	-	1.00*	140
Above average	-0.428	0.219	0.65	97
Self-financial asssment				
Less than Comfortable	0.212	0.165	1.24	113
Comfortable	-[a]	-	1.00	94
More than Comfortable	0.303	0.196	1.35	123
Method of recruitment				
Staff recruitment	0.197	0.365	1.22	118
Self recruitment	0.003	0.202	1.00	100
Chain recruitment	-[a]	-	1.00	99

[a] This symbol (-) refers to baseline category. No parameter is estimated for this category in the model.

[b] Risk factor represented by a single coefficient in model.

[c]SAP=(Attrition Probability/OAAP)X100.

[d]Calculated at the average time of follow-up (8.8 years).

*Likelihood ratio test on relative risks yields $p<0.05$. ***$p<0.001$.

was 97 increasing to 140 for subjects who rated their health as average or below. These values indicated a 3% reduction in the OAAP for subjects rating their health as above average and a 40% increase in the OAAP for subjects rating their health as average or below.

Figure 1 shows the percent attrition for years after initial visit for subjects with (1) maximum, (2) average, and (3) minimum effect of the eight risk factors on attrition. After the average follow-up time of 8.8 years, the percent attrition in the maximum group was 95%, in the average group was 22%, and in the minimum group was 5%. Figure 2 shows the relative contribution of each significant risk factor at 8.8 years to the percentage change in overall average probability for the maximum and minimum dropout risk groups. Subjects who enter the study living 500 or more miles from BLSA, at age 70 or over, with an education of less than a bachelors degree, and considering themselves as having average or lesser health, have the greatest increased probability of dropping out from the study. The increased probability for such a subject in comparison with a subject with an average probability was 316% (Fig. 2).

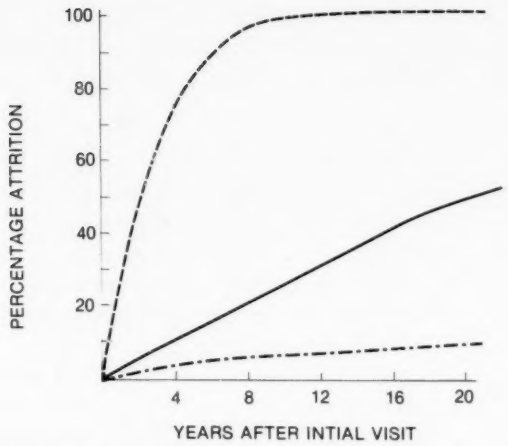

FIG. 1. Predicted effect of the eight factors, set at values which corresponds to Maximum (– – –), Average (——) and Minimum (– • –) risk levels, on attrition of BLSA subjects over time (1958–1977).

DISCUSSION

The results of the univariate analysis indicate that age, education and distance between a subject's residence and the study center are significantly associated with attrition. Possible strategies for the multivariate analysis of this data include the use of a modified logistic model and a proportional hazards model. Myers, Hankey, and Mantel (1973)

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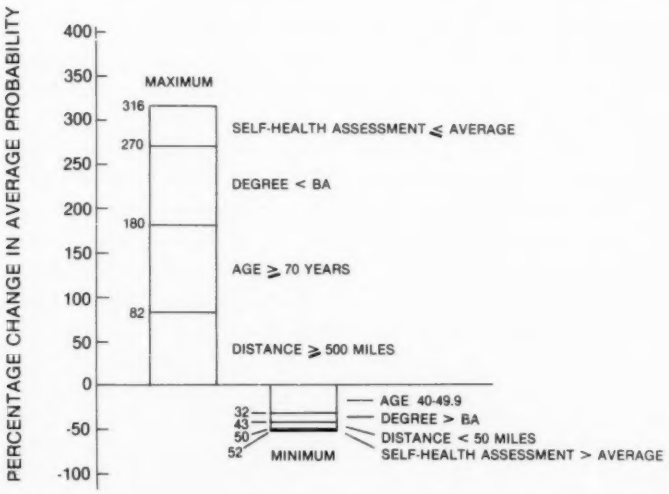

FIG. 2. Relative contribution of each significant risk factor for dropping out. The predicted probabilities are based on the study population's distribution for each factor and the average time of follow-up (8.8 years).

noted the limiting feature of a general logistic model when dealing with time-dependent outcomes. They suggested introducing an additional parameter of duration of time observed as a covariant in the logistic-exponential model in an attempt to compensate for this limitation.

The proportional hazards model treats the outcome variables themselves as time dependent. The coefficients obtained by this model appear invariant to the time of follow-up and the standard errors decrease with increasing follow-up time (Green and Symons, 1983). In this study data were analyzed using a modified logistic as well as proportional hazards model. Both methods gave similar results for each factor as indicated by significance levels. Results from the proportional hazards method are presented; this is theoretically the preferable methodology since the estimated survival probabilities based on the proportional hazards model are time dependent.

The proportional hazards analysis on our data shows that in addition to the three factors found significant in the univariate analysis, self-health assessment is also significantly associated with attrition. An examination of Table 2, for example, shows that the observed dropout rate among subjects under 30 years of age is less than the observed rate among those aged 40–49 years. This contrasts with the results obtained using the proportional hazard model where subjects 40–49 years of age were found to have the lowest risk of any age. This difference in results could be because of the simultaneous adjustment of the effects of several variables in the proportional hazards model. In studies where multiple factors have influence on outcome it is highly important to use a statistical model that can weight the relative importance of a number of factors, and to adjust for the influence of these other factors in data presentation. The proportional hazards model used in this study offers adjustment of any given independent variable for the effect of all other independent variables while accounting for time-scale variability of recorded events, thus providing a powerful means of bias reduction because of the simultaneous occurrence of these variables.

In the literature, a distinction has been maintained between voluntary and involuntary attrition. Attrition is considered to be involuntary when death is the reason for dropping out. For all other reasons, the term voluntary attrition is used. It has been consistently reported in the literature that older subjects are more likely to drop out (Riegel *et al.*, 1967; Rose *et al.*, 1976). Old age is also highly correlated with attrition due to death (Riegel *et al.*, 1967; Atchley, 1969; Rusin and Siegler, 1975; Wilson and Webber, 1976). When subjects who died while on active status (Group A) were excluded from the analysis, age became an even more significant factor (chi-square statistic with 5d.f. increased from 28.4 to 46.4). The risk of dropping out for a subject 70 years of age and over at first visit relative to subjects 40–49.9 years of age increased from 2.89 to 3.72, whereas the relative risk for a subject under 30 years of age at first visit decreased from 1.91 to 1.79.

Further, it has been reported that subjects who perceive their health as poor are more likely to be older, and older subjects are more likely to drop out (Maddox, 1962; Streib, 1966; Atchley, 1969; Powers and Bultena, 1972; Wilson and Webber, 1976). Older people are also more likely to have physical conditions/illnesses, and old people are more likely to drop out due to death. Therefore, if we exclude deceased subjects from the analysis, we would expect self-health assessment to have an even stronger association with attrition. In our study, when we excluded deceased subjects from the analysis the relative risks for the above average self-health assessment group relative to the average or below group fell to

0.56 (44% less) and 0.59 (41% less), respectively ($p < 0.01$). We believe that these differences from analysis 1 may be due to the fact that people who are assessing their health as only average or less are indeed sick and this affects their ability to return.

Rose and Bell (1967) in a theoretical paper suggested that subjects for long-term studies should be recruited from the same locality as the study center. Their rationale was, the closer the distance, the greater the participation on the part of the study subjects. Our study supports this hypothesis. The risk of dropping out increases with distance.

One final and yet highly important application of our proportional hazards model is in describing the probability of attrition for any group of subjects over the 20 year study period. Figure 2 indicates that subjects, who enter the study between the ages 40–49.9, with an education of more than bachelors degree, living less than 50 miles from BLSA, and considering themselves as having above average health, have the smallest probability of dropping out. The decreased probability for such a subject in comparison with a subject with an average probability was 52% (Fig. 2). An estimate of greatest/lowest drop out probability such as this can be particularly useful to the planners and the administrators of long-term investigations in effectively introducing strategies aimed at minimizing the attrition of subjects. These strategies could be applied either at the time of recruitment of study subjects (i.e., selecting those subjects who have a higher possibility of staying) or during the course of the study. Further, the results obtained may serve as a useful basis for the foundation of future recruitment strategies for longitudinal studies of aging.

These findings address a major issue concerning longitudinal studies which may be compounded by the problem of attrition, namely representativeness. Theoretically, if one wishes to assure sample maintenance, than these findings suggest that only those subjects who are middle-aged, healthy, educated, and who do not live far away from the research center should be selected. However, the consequences of such selection would limit the generalizability of the study findings.

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This problem is further compounded by the fact that in any longitudinal study with the progression of time, subjects get older, some become sick, and some may move, i.e. older participants may move after retirement to either warmer climates or closer to their children. If all changes were to result in a movement to categories of higher risk, then there could be an underestimation of the true effects as a result of a misclassification bias. The effect that subsequent changes in these variables may have on subjects retention is an empirical question worthy of attention in a future study.

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THE COMPARABILITY OF LONGITUDINAL STUDIES AND THE ROLE OF CLINICAL TRIALS IN NORMAL AGING RESEARCH

SAMUEL W. GREENHOUSE

Department of Statistics/Computer and Information Systems, Bldg. C, Room 315,
George Washington University, 2201 G St. NW, Washington, D.C.

Key Words: comparability of longitudinal studies, clinical trials, normal aging

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IN 1974, WHEN I left the National Institute of Child Health and Human Development, the Gerontology Research Center was still a component of the Intramural Research program of that Institute. Up until that time I was close to research in human aging. Since that time, unfortunately, I have only been involved peripherally and occasionally. Thus, I am unable to make any scientific contribution but rather will present some general remarks about Dr. Shock and about aging research.

There are two main impressions that I have of Nathan. One relates to his views of the problems of aging research in the early years of the 1940's and 1950's. Among all the pioneers of research in human aging in that post-war period, Dr. Shock was one of the most quantitatively oriented. He, as others, undoubtedly had some model, or as he referred to models, some "hypothesis" to direct his research interests. At the time they were mainly concerned with physiological aspects of aging. In the Wesley M. Carpenter lecture he delivered in 1955, Shock (1955) compared two sets of mortality curves: one between the United States and India and the other for the United States between 1900 and 1950. The plots related the logarithm of mortality rates to arithmetic age. He then noted that all four curves were linear after age 40 or 50 with approximately the same slope. (Actually the three curves for the United States have almost identical slopes indicating that for every yearly increase in age past 40 there is an increase in mortality of roughly 8.2% whereas the linear part of the curve for India past 50 years of age has a somewhat smaller slope indicating a smaller yearly percentage increase in mortality). Such observations on the relationship of mortality with age had been noted before. But Shock went on to state that "the fundamental biological problem is to determine the factors that influence the slope of the curves—to find out why there is a systematic increase in probability of death with increasing age."

Shock did not pursue further the implications on the kinds of studies that would take account of this observation that mortality past a certain age increases exponentially with age. Studies that immediately come to mind are those that obtain similar mortality-age curves for various well defined sub-groups of the population, defined either by behavior patterns, by life-styles or by high and low risk psychological and physiological

characteristics. For example, do joggers or vegetarians or individuals who are confined most of their lives in mental institutions show slopes similar to or different from that of the general population? Or are their mortality curves linear at all over any segment of their lifespan? Clearly, such studies are difficult to do. The data are probably not available now and would have to be collected over a 40 or 50 year period, longer than our longest ongoing longitudinal studies.

The second impression of Dr. Shock that I gained in those years concerned his tenacity and perserverance in fighting for a longitudinal study and for an NIH Institute on Aging. The longitudinal study came first but I cannot recall whether that was initiated while he was still in the National Heart Institute or after he had moved over to the NICHD. When I arrived at the NICHD in 1966 to establish a Biometry and Epidemiology Branch, one of the first Institute scientists who called was Dr. Shock. We discussed a number of methodological and administrative issues, the most urgent of which was a request for a full time statistician to be assigned to the Gerontological Research Center in Baltimore. Dr. LaVeck, director of the NICHD at the time, favored this request and I was able to recruit Dr. James Schlesselman who was most helpful to all the scientists at the Center and contributed significantly to the design and analysis of longitudinal studies. As we all know, the Center became the core of the intramural scientific program in the newly established National Institute on Aging with Dr. Shock as the Scientific Director.

There are two subjects I would like to mention relating to Aging Research generally. The first has to do with longitudinal studies. Recently, I wanted to obtain data showing the distribution of Ejection Fraction (EF) values by age. More precisely, I wanted the percentage of healthy individuals having EF values below 45 (and also 40 and 50), critical values customarily used by cardiologists to determine poor cardiac function. I thought the most convenient sources would be the publications of the major longitudinal studies. I turned to the 1984 publication of the Baltimore Longitudinal Study of Aging (Shock *et al.*, 1984). This volume presented a chart relating an EF index to age which was not useful at all. In fact, the EF index did not seem to bear any relationship to the clinical EF measurement and the text did not discuss the nature of the index at all. I then referred to two books reporting on the Duke Longitudinal Study (Busse and Maddox 1985, and Normal Aging III 1985) and found no reference to EF measurements. Clearly, the Duke investigators did not include exercise tests in their examinations.

This episode raised a rather troublesome question. A number of major longitudinal studies were undertaken to study normal aging employing different populations of subjects. It is obvious that the investigators directing these studies may have had different objectives and different emphases of the variables to be examined. However, given the tremendous effort in resources and time these studies required, can they not be used to replicate findings from one study to another? For example, are the cognitive changes over time (longitudinally) found by the BLSA the same as or different from those found by the Duke study? And how do these compare with cognitive variations due to aging in other studies? Wouldn't it be fruitful for a small group of investigators representing the various major longitudinal studies to get together and review those variables in each of the major disciplines (physiological, psychological, etc.) to determine which are comparable across studies. The next step would clearly be to examine two questions: (1) do these common variables show the same longitudinal pattern or in what way do the patterns differ; (2) are

relationships between variables (e.g., the effects of physiological changes on cognition and behavior, etc.) similar or are they different?

In recent years there has been an increased interest in comparing results in independent studies exploring the same issues. The field is known as meta-analysis. Statisticians and others have developed methodologies going far beyond Fisher's original procedure for combining P-values from independent studies. Most of the applications of meta-analysis in educational research, in the social sciences and in clinical research are designed to enhance the chances for finding statistically significant results. However, the application of meta-analysis procedures to independnt longitudinal studies on Normal Aging may have objectives other than those related to hypothesis testing. One is a simple descriptive analysis of comparing patterns of change over time in different human samples. Another would be to compare relationships between variables among the various studies. A third might be to compare high risk factors for abnormal aging in any sense among the different studies. Although the major studies were not designed to attain uniformity and comparability of results among them, the analyses proposed would in my view be an appropriate attempt to gain the maximum information from these complex, time-consuming projects.

The second topic relates to the dearth of randomized clinical trials in aging research. It is surprising to note that, as far as I know, there have been no major trials engaged by gerontologists. Is it because there exists no important issue concerning the prevention of certain aspects of abnormal aging? I exclude therapeutic trials. These trials involving elderly patients already exist or have been done in the cardiovascular, pulmonary, gastrointestinal, and opthalmological areas. Thus the aged become subjects in clinical trials only because they have diseases. Furthermore, the trials are conceived and directed by specialists in those diseases. These trials are clearly not to be considered studies of normal aging.

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It is of interest to point out that a randomized, controlled, double blind clinical trial was conducted in England (Miller, 1969) pertaining to the treatment of osteoporosis. It was a relatively small trial involving 38 patients randomly allocated either to a control group receiving calcium supplements and Vitamin D or to the treatment group receiving the same substances as the control plus a sex hormone. The investigator concluded, "there appears to be little if any additional relief of symptoms of osteoporosis when treatment of the osteoporosis with calcium lactate and Vitamin D is supplemented by the use of sex hormones." One must comment that there probably was very poor statistical power to detect any significant improvement if sex hormones were effective.

The treatment of osteoporosis could be considered as research more closely connected with normal aging. Yet osteoporosis is still a disease in the medical sense. But then what are the issues that can be thought of as truly problems in normal aging. I can propose one although I am sure that gerontologists can offer many more. Whether preventive programs exist so that a clinical trial is appropriate at this time is another matter.

Arenberg (1983) has presented data from the Baltimore Longitudinal Study of Aging indicating that memory and learning do decline in later life and that the deficits beginning at age 60 are quite large. These results apparently contradict the observations made by other investigators. (I suspect, however, that after taking account of differences in subjects, in tests and in how the tests were conducted, contradictions would disappear.) If Arenberg's findings hold up then this could lead to a prevention clinical trial in normal aging, assuming that some training program or other agent were available and thought to be potentially ef-

fective. Furthermore, although I previously excluded therapeutic trials, Arenberg proposed an area of research "to devise mnemonics and other training programs specifically for the aged to minimize deficits in learning and memory". As phrased, I interpret him to offer this as a possible treatment for those who have already demonstrated some degree of decline in these functions. If this were to be tested by a randomized, controlled clinical trial, it would constitute a therapeutic trial.

One does not have to be a scientist engaged in research to be aware of the many programs related to diet, exercise and other proscriptions for better living that have never been adequately evaluated. At the same time I must caution that a randomized clinical trial is not any easy activity to conduct. It is possible that an area may not be ready for it. It is well known that diet studies are difficult to do. The treatments to be used in the treated and control group must be well defined; eligibility criteria for entry into the study specified; baseline variables, frequency of visits and measurements to be made at follow-up established; outcome variables precisely defined and a data handling unit established. Despite the complexity inherent in a randomized clinical trial, it is now recognized as the most definitive methodology for evaluating a new therapy or prevention program either against a placebo control or a standard treatment control. I would imagine that among the many problems associated with normal aging there must be some related to choices to be made by the elderly or those in middle age or choices to be made by clinicians in advising patients for which there is little or no evidence favoring one alternative or the other. If gerontologists and geriatricians can develop some sharp hypotheses about the efficacy of these alternatives in prevention or in treatment, then they should seriously consider randomized clinical trials as the most effective way of testing these hypotheses.

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MECHANISMS OF AGE-RELATED ALTERATIONS OF HORMONE SECRETION AND ACTION. AN OVERVIEW* OF 30 YEARS OF PROGRESS

ROBERT I. GREGERMAN

Gerontology Research Center, National Institute on Aging, National Institutes of Health at Francis Scott Key Medical Center, Baltimore, Maryland 21224

Key Words: aging, hormone secretion, hormone action, membranes, receptors

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IN TAKING pause to do honor to a man who has had a determining impact on the development of gerontology as a legitimate area of scientific inquiry, I realize once again that Dr. Nathan W. Shock has also had a mighty influence on my own fate. The year 1986 marks the 30th anniversary of my arrival at Dr. Shock's creation, the Gerontology Branch, as it was then known, and my introduction to the field of aging. I certainly did not bring many prejudices into this venture, since when I first heard of the man and the place, I had no idea that anyone, anywhere, was interested in such matters. Nor did I give any deep thought to the potential impact on me—let alone on the emerging field—of my first gerontologic investigations which Dr. Shock inspired and would one way or another determine my path for the next three decades. Now that I am here, however, I am pleased to be able to record some observations about aging and hormones and to dedicate this overview to the man who invited me into this field. Dr. Shock has shaped my thinking in ways which I now realize are both obvious and subtle.

For me the beginning of gerontology was in the year 1956. By that time little enough was known about the effects of aging on hormones—or hormones on aging—to make reviews of the topic very easy indeed. But, before we come to specifics, I would like to make some observations that may be informative to those who wish to learn from the mistakes of the past.

This area is now known to be a heterogeneous one in which age-related alterations of hormone secretion and of hormone action in both man and animals occur through a variety

*Our library's dictionary (*Webster's Third New International Dictionary*, G.C. Merriam Co., 1976) defines an "overview" as a general view or survey. The example of usage for this word is given as follows: "A quick overview tempts one to make a few generalizations-Frances Spain." I incorporated this word in the title to connote precisely this meaning and in an attempt to disclaim the notion that I have written anything resembling a comprehensive review. As for usage, I have certainly succumbed to the temptation of making generalizations.

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of mechanisms. Some of the alterations of hormone secretion are already known to have significant clinical implications; for others, clinical implications are only suspected but are of great potential importance. In many situations, age-related disease processes result in alterations of hormone secretion which are then phenomena secondary to disease, rather than aging, per se. In some of these latter instances, the effects of disease can be mistaken for aging. One has also to keep in mind that old animals, like old people, may also be sick. For a variety of reasons, age-related alterations of hormone action are known mainly from animal models, although some of these will undoubtedly prove to have implications for human physiology, as well.

Certain corollaries of these statements are worth considering. Many investigators enter into their work in this area with the notion that studies in animals can be readily extrapolated to man. In the field of hormones and aging, nothing could be further from reality. If an age-related change is due to an alteration of hormone metabolism, the extrapolation is particularly suspect. All one can say after demonstrating a change with age in an animal "model" is that such a change occurs in the species examined and that comparable studies in the human *may* be worth performing and *may* conceivably yield parallel results. It is a constant source of amazement to me that authors are still writing—and editors still accepting—that their latest finding in the rat has direct impact for thinking about human age-related changes. On the other hand, I hope that my view of this subject will not be misinterpreted. I do appreciate the importance, or rather the essentiality, of animal work in this area of research, and I am certainly not against research using animal models or tissues. Indeed, I spend much of my research effort engaged in precisely such activities. I merely advocate a conservative approach to the process of extrapolation of research findings–at least in this area—from one species to another. Another point which is now recognized by informed and seasoned gerontologists but is hardly known to other scientists—including some with outstanding expertise in their own areas—is that the word "aging" conveys different meanings to different people. Those of us who were influenced by Nathan W. Shock believe that it is useful to distinguish between growth and development, the process of maturation, and the changes that accompany senescence. We are not always dealing with a continual process. Thus, large changes in hormone secretion or action usually occur during growth and maturation. One cannot always look at results in young (2–3 month old) rats and compare them with those of senescent animals (24 months or more) and say anything sensible, at least as one conventional school of gerontologists considers the problem of aging. The difficulty with such a comparison is that since no mature (mid-life) group is included (6–12 months for the rat), one cannot know whether an observed difference between groups is attributable to aging (senescence) or maturation, a process which is frequently associated with large changes of function. On some occasions in which it is known that the phenomenon at hand changes progressively over the lifespan, studies of short intervals of time over a portion of the early life of the animal may be appropriate and, of course, have practical advantages to the investigator. It remains to be seen whether increasingly frequent warnings on this point (e.g. Gregerman, 1983; Florini and Regan, 1985) will have the desired effect of eliminating this type of error from the literature.

The considerations that follow below are no attempt at a comprehensive review of all of the reports that deal with the subject of hormones and aging. Rather, I have attempted to identify some broad categories of age-related changes of hormone secretion and action and have illustrated them by specific examples. One can be certain that, as time passes,

additional mechanisms will become apparent. For the reader interested in this area of the literature, a number of general reviews are available (Gregerman and Bierman, 1981; Korenman, 1982; Dax, 1985; Florini and Regan, 1985; Minaker et al., 1985; Blackman, 1986).

In addition, other reviews are available that deal with more specialized areas such as reproductive endocrinology (e.g., Harman and Nanin, 1985; Harman and Talbert, 1985). Aspects of the topic are also included in related reviews such as those on mineral metabolism (Exton-Smith, 1985) and cardiovascular topics (Lakatta, 1985). The reader should note that this overview hardly mentions topics within the field of neuroendocrinology (Finch and Landfield, 1985; Rogers and Bloom, 1985). It is also obviously impossible and certainly not desirable to ignore central nervous system neurotransmitters (Rogers and Bloom, 1985) which are considered by many to be hormones, albeit those which are secreted and act locally (e.g. the hypothalamus). But where does endocrinology begin or end? One person's hormone is another's neurotransmitter. With sincere apologies to any who are offended by these omissions of active and fascinating areas, I hasten again to retreat into our dictionary and its definition of "overview".

ALTERATIONS OF HORMONE SECRETION

Failure of hormone secretion

Occasionally, aging results in nearly total cessation of hormone secretion. The best example of this type of age-related change is failure of development of the ovarian follicle and the resultant decrease of estrogen secretion by the ovary. This event heralds the onset of menopause in all women, a period of time which is often accompanied by a variety of psychologic symptoms and autonomic instability that leads to disturbing episodes of upper-body vasodilatation (flushing). These events are clearly triggered by estrogen deficiency. A serious long-term consequence of failure of estrogen secretion is bone mineral loss. Eventually this often results in clinically manifest osteoporosis with spontaneous collapse of vertebrae and a propensity to fracture of major bony structures (hip, wrist). Discussion of the pathogenesis and therapy of this estrogen deficiency state with its accompanying malabsorption of calcium, loss of estrogen's effect on conversion of 25-hydroxy-vitamin D_3 to 1,25-dihydroxy-vitamin D_3, loss of estrogen effect on bone, and atrophy of hormone-responsive soft tissue is beyond the scope of this brief review. However, it is clear that this change of hormone secretion can be a significant one in the later decades of life of the human female (Gregerman and Bierman, 1981).

Decreased hormone secretion rates secondary to altered metabolism

A bit of reflection will bring home the point that the concentration of a hormone in blood is the result of both secretion rate and degradation rate as well as the binding of the hormone to carrier proteins in plasma. Clinicians tend to equate hormone concentration ("level") in blood with secretion rate and work on the premise that the higher the hormone level, the greater the secretion rate and vice versa. Thinking along this track is acceptable for most clinical purposes, i.e. gross disturbances of secretion rate in which degradation rate does not vary appreciably and the binding of hormone to carrier proteins is either not changed or can be taken into account. However, during aging, significant alterations in hormone degradation rate may occur leading to a homeostatic decrease of secretion rate

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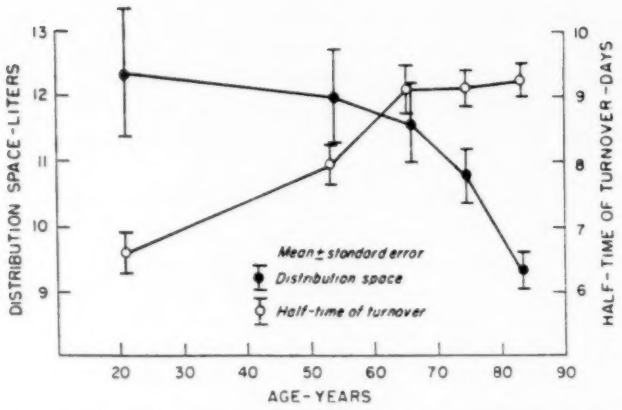

FIG. 1. RELATIONSHIP OF AGE TO THYROXINE DISTRIBUTION SPACE AND HALF-TIME OF TURNOVER, BY AGE GROUPS. Total number of subjects, 73. The lines connecting means are drawn by inspection. The vertical lines represent one standard error above and below the mean for each group. The half-time of turnover increases with age, i.e. the fractional turnover rate (not shown) decreases. From Gregerman *et al.* (1962).

which is necessary to keep the blood hormone level from becoming elevated. Thus, it is of interest whether the blood level of a hormone changes with age, but that change, or lack thereof, may not reflect the secretion rate. Despite the elementary considerations involved in this statement, some investigators persist in reviewing the literature which reports little or no change in blood hormone level and then erroneously conclude that there appears to be relatively little change in gland function with age.

As early as 1956 it was apparent that thyroid hormone (thyroxine) level in blood changed little with age, but thyroidal radioiodide accumulation rates and plasma clearance decreased. The inevitable conclusion that the rate of thyroxine disposal must therefore be slowed was confirmed in direct measurements of the kinetics of thyroxine disposal (see Fig. 1; Gregerman *et al.*, 1962). The calculated decrease of thyroxine degradation, equal to synthesis in the steady state, indicated a change of about 50% over the adult age span (Fig. 2). This work has been confirmed and its therapeutic implication—that the replacement dose of thyroxine should be less in elderly individuals—has been demonstrated in recent studies (Rosenbaum and Barzel, 1982; Davis *et al.*, 1984). In these investigations the amount of orally administered thyroxine needed to restore the elevated thyrotropin (TSH) of hypothyroid individuals was shown to decrease with increasing age, as would be expected from the earlier kinetic studies.

Slowing of thyroxine disposal rate with increasing age is probably related to decreased activity of enzymes that deiodinate and otherwise inactivate thyroxine, although the precise factors that control this alteration of disposal rate are little better understood now than they were 30 years ago. However, the slowed disposal mechanism is not a fixed accompaniment of aging, since disposal rate can be increased in the elderly in response to acceleration of thyroid hormone metabolism such as occurs during the stress of infectious illness (Gregerman and Solomon, 1967). A more extensive review of this subject is available (Gregerman, 1986).

Although an age-related alteration of hormone secretion via slowed disposal has been

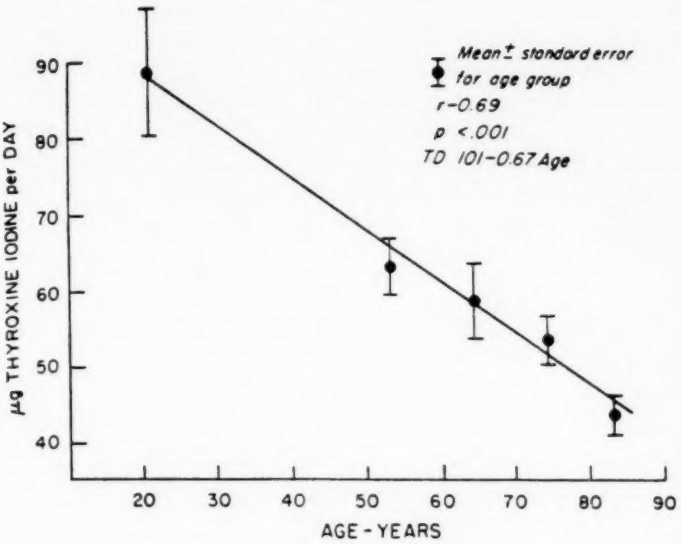

FIG. 2. RELATIONSHIP OF AGE TO DAILY THYROXINE DEGRADATION BY AGE GROUPS. The regression line is calculated from the regression analysis based on individual values for 72 subjects. The vertical bars represent one standard error above and below the mean for each group. From Gregerman *et al.* (1962).

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best studied for the case of thyroxine, cortisol and possibly testosterone disposal are similarly affected during aging. Other hormones may also be affected, but information along these lines is not presently available.

While much has been made in the past about the significance of plasma binding of hormones—the "free hormone" theory—recent work has questioned the significance of plasma binding as a determinant of overall hormone action. Nonetheless, plasma free hormone concentrations are determined in part by the concentration of their carrier proteins. In the case of thyroxine, the free hormone level is not affected during aging in man while variable changes are reported in animals (Gregerman, 1986). Thyroxine-binding globulin (TBG), the major protein carrier of plasma in the human, is not significantly affected by aging and does not appear to contribute to the minimal (10%) but statistically significant drop of T_4 that can be seen in some population (Harman *et al.*, 1984).

Decreases of hormone secretion due to alteration of pulsed release

Traditional studies of hormone secretion determine blood levels in a basal state or following stimulation with a tropic hormone. However, some hormones are secreted in pulsatile fashion. For these, static measurements of basal levels are not meaningful. Rather, both the frequency and duration of pulsed secretion are important in studies of both secretion and hormone action, since the latter may be largely determined by either the totally "integrated" blood level or some function of time and duration within the peak of secretion.

It is now apparent that the secretion of growth hormone is episodic. In young men,

about three-fourths of the pulses occur during the first hours of sleep. During aging in the human, these nocturnal pulses are greatly decreased in both frequency and amplitude (Fig. 3; Prinz *et al.*, 1983). Similar age-related changes occur in the rat. The biological effects of growth hormone are now believed to be mainly mediated via the stimulation of the hepatic production of somatomedins, especially somatomedin-C, an insulin-like growth factor. Circulating levels of somatomedin-C decrease with age, an event which is almost certainly secondary to decreased levels of growth hormone and with possibly important physiologic implications (see below).

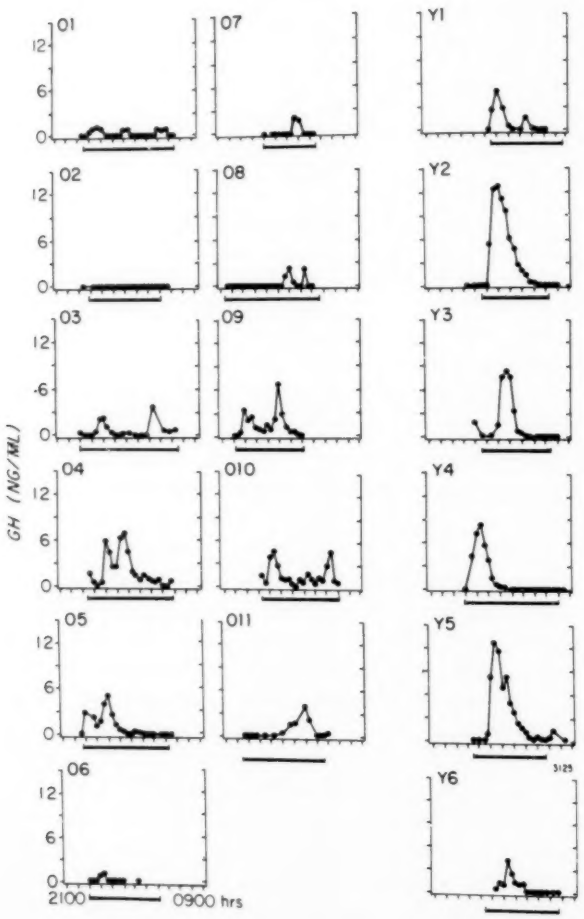

FIG. 3. Plasma growth hormone concentration (ng/ml) during the night in old (01 and 011) and young men (Y1 to Y6). The black bar indicates time in bed. Although considerable individual variation is obvious, some of the elderly men secrete very little growth hormone and their secretion peaks are reduced in amplitude. From Prinz et al. (1983).

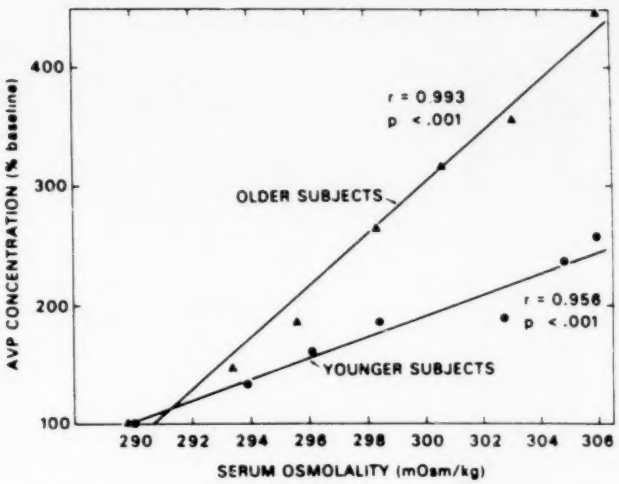

FIG. 4. Correlation between serum osmolality and AVP concentration in young and old subjects during a 2-hour intravenous infusion of 3% NaCl at the rate of 0.1 ml/kg per min. The points represent mean values of osmolality and AVP at successive 20 min in each age group. Increased secretion of antidiuretic hormone (ADH; vasopressin) in response to osmolar loading in elderly subjects. From Helderman *et al.* (1978).

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Altered set points of physiologic regulatory centers

Aging can affect the sensitivity of physiologic regulatory centers. A striking example is the hypothalamic-pituitary regulation of secretion of antidiuretic hormone (ADH; vasopressin). Several alterations of water metabolism may result from this phenomenon. Some years ago the syndrome of drug-related inappropriate secretion of anti-diuretic hormone was noted in elderly individuals receiving chlorpropamide (Weissman *et al.*, 1971). In addition, disturbances of ADH secretion have been reported in patients subjected to stress of surgery and anesthesia. These observations led to the suspicion that old people might have a disturbance of ADH regulation. Subsequent studies of ADH release in young and old individuals in response to stimulation by hypertonic NaCl and the ability of ethanol to inhibit ADH secretion confirmed these suspicions. Stimulation of ADH secretion by osmolar loading is increased in old persons relative to young (Fig. 4), implying an increase in the sensitivity of the osmoreceptor in the old (Helderman *et al.*, 1978). Studies of the ability of ethanol to inhibit ADH release in the elderly show a decrease in the ability of ethanol to suppress ADH release, but this may be the result of override of the osmostat by the greater sensitivity to ethanol's osmolar stimulation (Fig. 5). In any case, the set-point of the physiologic receptors in the hypothalamus that regulate ADH secretion are clearly affected during aging. On the other hand, volume-pressure sensitivity as estimated by the response to orthostasis shows decreased responsiveness in the elderly. This change is thought to result from a defective baroreceptor reflex arc somewhere between the vasomotor center and the hypothalamic supraoptic and paraventricular nuclei (Rowe *et al.*, 1982). In addition, recent work suggests altered rhythms of ADH release and electrolyte excretion during aging. In this report it was clearly shown that whereas young per-

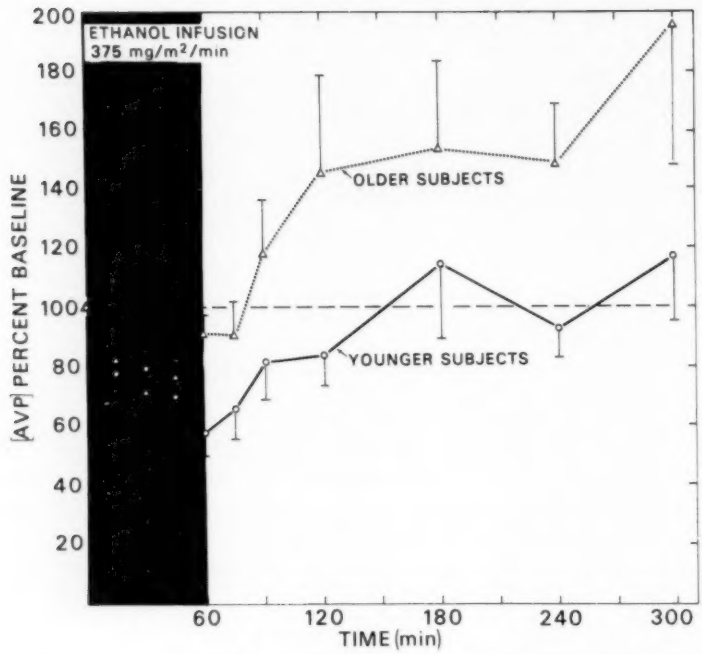

FIG. 5. Plasma arginine vasopressin response to a 1-hour intravenous ethanol infusion in young and old subjects. The AVP concentration after overnight dehydration and prior to the study is designated 100%. The AVP is significantly depressed from the baseline concentration in the young group from 15 min through 75 min ($p < 0.02$, 0.05, 0.025, 0.005, 0.02, respectively). Only the 15-min and 30-min concentration in the older subjects were significantly altered ($p < 0.05$). Suppression of antidiuretic hormone (AVP) is less in older than in young subjects during infusion of ethanol (1st 60 min). Thereafter, older subjects secrete more AVP, perhaps because of their increased responsiveness to osmolar stimulation (see text and Fig. 4). From Helderman *et al.* (1978).

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sons excrete twice as much urine during the day as at night, the elderly excrete nearly the same urine volume during the day as at night (Kirkland *et al.*, 1983). A clear explanation for this phenomenon is not yet available, and direct measurements of possible diurnal variations of secretions of antidiuretic hormone have not yet been reported, but age-related alterations of ADH secretion may well underlie the altered pattern of urine output so commonly seen with aging. Studies of this type may help explain the very common clinical phenomenon of nocturia.

Already available evidence suggests other age-related alterations in hormone-responsive hypothalamic centers. Although experiments in which pharmacologic doses of infused hypothalamic releasing hormones have in man usually failed to reveal evidence for age-related alterations of pituitary responsiveness, such changes may, nonetheless, exist under physiologic conditions. Thus, physiologically adequate amounts of estrogen replacement are inadequate to return postmenopausal gonadotropins to their premenopausal levels, suggesting an alteration of the set-point of the hypothalamic centers that control this negative feedback loop. This issue deserves more careful study, however, and will require

experimental protocols that adequately reproduce normal blood levels of naturally-occuring estrogens. Similar observations with regard to an age-related change of set-point have been made in the male with some studies reporting elevated levels of gonadotropins with unchanged daytime levels of circulating testosterone. The elderly, however, seem to have lost the normal rise of testosterone in plasma that occurs between 1300 and 0100 h; the mechanism of this phenomenon is not presently understood (Bremner *et al.*, 1983). Once again, blood hormone concentrations at a fixed point in time give only an incomplete and misleading picture of hormone homeostasis in the elderly.

Selective acquired defects of hormone synthesis

A few instances of age-related alterations of endocrine gland responsiveness are highly selective within a given organ. The adrenal normally produces three classes of hormone: glucocorticoids (principally cortisol), mineralocorticoids (principally aldosterone), and a variety of so-called adrenal androgens (mainly dehydroepiandrosterone, DHEA). During aging in the human, responsiveness of cortisol to stimulation by ACTH is unimpaired, but the gland progressively decreases its output of DHEA and also loses its ability to respond to ACTH with respect to this compound, presumably a result of an acquired enzymatic defect(s) in the synthetic pathway. Aldosterone secretion is greatly diminished, presumably secondary at least in part to decreased secretion of renin which is in turn responsible for the generation of angiotensin, a direct stimulus to aldosterone secretion. Circulating levels of aldosterone are decreased in the elderly by as much as 60–70% after sodium restriction and may contribute to the clinical phenomenon in the elderly of impaired ability to retain sodium when deprived of the ion (Epstein and Hollenberg, 1976).

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The ability of the adrenal of the elderly to produce glucocorticoids under basal circumstances, in response to ACTH infusions, and even in response to endogenous ACTH secreted under the stimulus of stress has been repeatedly and very well documented. As expected, the secretion of ACTH in response to infusion of corticotropin-releasing hormone (CRF) is also maintained in the elderly (Pavlov *et al.*, 1986a). A small time-shift (phase-advance) in the diurnal rhythm of cortisol has been recently reported (Sherman *et al.*, 1985). In contrast to this situation of preserved function in the human, a variety of age-related alterations of responsiveness to ACTH have been reported for isolated adrenal cells of the old rat. Although earlier work with intact animals had suggested little or no effect of age, a recent study using isolated adrenal cells showed marked age-related changes. Maximum responsiveness of corticosterone to ACTH was decreased and the sensitivity of ACTH was also diminished (Malamed and Carsia, 1983). On the other hand, yet another recent study indicates maintenance of circulating corticosterone with increased responsiveness to diminished secretion of ACTH in one part of the day (Sonntag *et al.*, 1986). Such variable and diverse reports for the same species speak clearly to the issue of the difficulty of using animals as models for age-related changes in man!

Effects of disease on hormone secretion: misattribution to aging

The effects of disease—sometimes clinically subtle—appear to contribute to what is at first thought to be "normal aging". Early reports of age-related changes of serum testosterone showed large decreases with age and reciprocal changes (elevations) of gonadotrophins. These were thought to represent phenomena of aging due to age-related gonadal failure. Later studies of carefully screened healthy men showed no such age-related

decreases of plasma testosterone. Recent studies of sick elderly men appear to indicate that illness affects gonadal testosterone output and emphasize again the need to take clinical status very carefully into account before attributing endocrine changes to aging, per se (Harman and Nankin, 1985).

The history of the story of decreased circulating levels of triiodothyronine (T_3) during aging is another example of how the effect of illness can be misattributed to aging. Early reports indicated a very large decrease of circulating T_3 in elderly persons. These studies included the elderly sick until it became apparent that most of the circulating T_3 is derived by deiodination of thyroxine (T_4) and that this process is very easily impaired by decreased food intake such as occurs during almost any form of illness. The result is decreased circulating T_3. When elderly persons are carefully screened to exclude illness, either no decrease of T_3 is seen or a decrease of small magnitude (10%) may be apparent (Harman *et al.*, 1984; Gregerman, 1985). The physiologic significance of such a small change is not usually apparent.

Changes of hormone secretion occuring secondary to altered function in another organ

Secretion of hormones may be influenced by age-related events in distant organs. One example, already presented, is the homeostatic decrease of thyroxine secretion which follows age-related slowing of disposal via metabolic destruction in the liver. Another example of this type of change apparently occurs with parathyroid hormone secretion, but the sequence is different. Secretion of parathyroid hormone (PTH) appears to increase with increasing age. One of the first reports indicated a huge increase of PTH in plasma and suggested a large increase of secretion in elderly persons, especially women. This finding was thought to be of great significance in considering the pathogenesis of osteoporosis, but enthusiasm was short-lived. Others saw no such dramatic increase, and it became apparent that some technical aspect of the immuno-assay procedure used for the measurement of PTH was responsible. In many of the early radioimmunosassays for PTH the antibodies employed reacted with the C-terminal fragment of PTH arising from the hormone's degradation. Since renal function decreases with increasing age, it was then suspected that accumulation of C-terminal fragment due to impaired excretion and/or metabolic destruction in the kidney could explain the apparent increase of PTH with age. Unexplained was the far greater increase of PTH during aging in women than in men, although later work showed that while renal function decreases with age in both men and women, the decrease is greater in women. Accordingly, the first impression of a major increase of biologically active PTH during aging could not be substantiated. However, when studies of PTH levels were made using antibody which recognizes intact PTH or the N-terminal but not the C-terminal fragment, a modest increase of PTH was still observed (Marcus *et al.*, 1984). Moreover, there was evidence for increased PTH activity on the renal handling of phosphate and generation of cyclic AMP, suggesting that the increased immunoreactive PTH was indeed exerting an increased biological effect, at least on the kidney. One reservation remains in considering these results. The estimates of immunoreactive PTH may still exceed those of biologically active material, since the antibody recognizes some fragments of PTH-other than the C-terminal ones—that are also biologically inert. With this caveat in mind, it would appear that decreasing renal function with age may well result in increasing secretion of parathyroid hormone with metabolic

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consequences for calcium homeostasis. This rather tenuous and hesitant conclusion comes at the end of nearly a ten-year saga of the vagaries of modern endocrinology with its invaluable, but sometimes misleading, immunoassays. It would be difficult to find a better example of the difficulties of unraveling an aparently simple issue.

AGE-RELATED ALTERATIONS OF HORMONE ACTION

Advances in our understanding of how the action of hormones is affected by age is very recent, and our knowledge of the mechanisms involved is just beginning. However, we are now in an era when almost as soon as new areas of research interest appear the age variable is examined and, quite often, quickly found to be involved. In contrast, the process of understanding the biochemical mechanisms of age-related changes is much more difficult and proceeds at a very slow pace. Needless to say, the issue of age-related alteration of hormone action is usually more easily studied in animals, although clinical investigations sometimes give the first clue to the existence of an age-related event.

Receptors and age-related alterations of hormone action

The past decade has seen an enormous interest in the area of hormone receptors. In part the abundance of data on receptors reflects the ease of performing studies which involve "grinding and binding" experiments. Unfortunately, for many receptors, little more than a catalog of age-related changes has ensued, with some of the data flawed by technical problems serious enough to invalidate the results. As the years have passed, it has become apparent that those who wish merely to apply "standard" techniques to problems of aging are likely to be misled by experimental and methodological artifacts. With this serious reservation one can generalize—rather uselessly, perhaps—that a number of systems show decreases of receptors during aging. Others show no change and a few show increases (Roth and Hess, 1982; Dax, 1985). Few, it any, conclusions should be drawn from studies which deal only with receptors, since hormone action is much more complex than the number of hormone receptors on a cell membrane or within the cell's interior. Furthermore, the number of receptors of cell surfaces is not fixed, but varies rapidly with the hormone concentration in the cell's milieu. The ability of cells to regulate receptor number through synthesis or redistribution may also vary with age, although only a few observations are available to date (Dax, 1985).

More informative than identifying receptors that change with age are studies which attempt to correlate receptor number (or affinity) with some biological or biochemical event. The number of such studies is quite small and deal with only a few of many known hormone-sensitive events. In some situations, the change of receptor number may well account for the change of biological response. However, in other cases, receptor numbers have been measured and do not appear to change even though the evoked response is clearly decreased. Such events are interesting, but their description by the collective term "post-receptor defects", though useful jargon, exudes a certain unhealthy pomposity by implying that we understand something when we really understand very little. The variety of hormonally mediated responses that are affected during aging is obviously very wide and must involve a whole array of "defects". Some of these events deserve special mention.

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Insulin and carbonhydrate tolerance

For many years it has been known that the ability of man to dispose of a glucose load is impaired during aging, i.e. when the concentration of glucose in blood is increased by oral ingestion or intravenous infusion, the metabolic disposal of glucose is slower in the old than in the young. The distinction of this phenomenon—which affects half of individuals over the age of 60—from diabetes mellitus is an interesting exercise which we shall avoid, since others have more courage (Elahi *et al.*, 1984; Minaker *et al.*, 1985; Reaven and Reaven, 1985). Suffice it to say that, although diabetes mellitus also appears to increase with increasing age, it seems unlikely that diabetes affects one-half the population. In the elderly, the pancreatic secretion of insulin in response to glucose loading is not greatly affected; plasma levels of insulin are actually increased beyond that expected for the degree of glycemia and presumably reflect some decrease in the rate of metabolic clearance of insulin and/or increase in insulin secretary capacity per pancreatic β-cell. A number of studies indicate that the slowed metabolic clearance of glucose is mainly the result of blunted responsiveness to the metabolic effect of insulin, an event which is "post-receptor". In the parlance of the glucose/insulin investigator or the clinician, aging is associated with the development of insulin resistance (Reaven and Reaven, 1985). The multifactorial aspect of this phenomenon is shown by the demonstration that the pancreas' response to the enteric mediator which stimulates insulin release (gastric inhibitory peptide [GIP], the major insulin secretagogue) is also blunted by aging (Elahi *et al.*, 1984). Moreover, even though body composition (percent fat vs. muscle) and physical activity are clearly factors affecting insulin action during aging (Reaven and Reaven, 1985), the exact biochemical mechanism of age-related insulin resistance is unknown.

Thyroid hormones

Thyroid hormones appear to have multiple effects. Control of the rate of tissue metabolism has been the longest known of these, but the precise mechanism(s) of action of the thyroid hormones are only recently beginning to be understood. Thus, thyroid hormones conrol gene expression and thereby the synthesis of specific proteins such as enzymes, but also appear to affect membrane function through direct effects on the components of membranes including certain key enzymes (e.g. sodium-potassium and calcium dependent ATPases). Although some work has suggested that aging may affect the tissue availability or action of thyroid hormone in control of tissue respiration in animals, no clear picture has emerged (Gregerman and Bierman, 1981; Sartin, 1983). One related point that was made very clear by Dr. Shock and his colleagues cannot be ignored here. The decrease of basal metabolic rate with age—once thought to be due to altered thyroid function or action—was shown instead to be due to a change of body composition (Fig. 6; Gregerman and Bierman, 1981).

Thyroid hormone action in the human seems to be strongly influenced during aging, although the process defies quantitation. When thyroid hormones are secreted in greater than physiologic amounts, the disease state termed hyperthyroidism ensues. In the elderly, the activation of sympathetic nervous system activity that is characteristic of this disease state is grossly blunted so that the manifestions of hormone excess are much reduced. Attempts to explain this phenomenon on the basis of imparied effects on the induction of β-adrenergic receptors or receptor:adenylate cyclase interactions are not presently convincing for the human (Gregerman, 1985).

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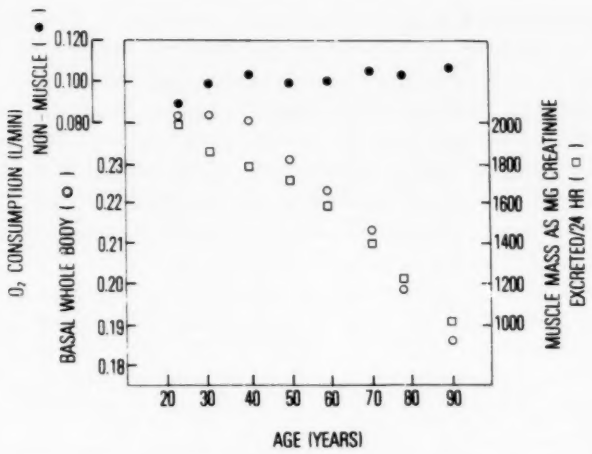

FIG. 6. Basal oxygen consumption (BMR) and age in male subjects. Note decrease of whole-body oxygen consumption with age (○). Muscle mass, in this study estimated from creatinine excretion (□), also decreases with age. Oxygen consumption calculated per unit nonmuscle metabolic mass (●), however, shows no such decrease. Thus the age-related decrease of BMR is considered to be a function of altered body composition with age rather than a result of diminished thyroid function. Similar age-related decreases of muscle mass can also be shown with measurements of intracellular water or of body potassium. All of these estimates (see also Fig. 29-2) decrease with age, (Modified from Tzankoff, S.P., and Norris, A.H.: Effect of muscle mass decrease on age-related BMR changes. *J. Appl. Physiol. 43:*1001, 1977.) From Gregerman and Bierman, (1981).

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Glycosylated hormones

An interesting mechanism by which aging can affect hormone action is through an effect on protein synthesis of certain hormones. The glycoprotein hormones of the pituitary become glycosylated and biologically active only after the protein portion is synthesized. Evidence exists to indicate that glycosylation of these hormones may be impaired during aging. Thus, hormone action may become impaired as well. Since most hormones are measured by immunoassay, altered biological activity may exist while immunological activity may be unaffected. An example of this phenomenon is already evident with luteinizing hormone (LH) where discordance between biological and immunological activities occurs during aging (Marrama *et al.*, 1984; Warner *et al.*, 1985). The measurement of all glycoprotein hormones by immunoassay is now suspect in any state—including aging—in which glycosylation might be impaired.

Growth hormone and somatomedins

As mentioned earlier, growth hormone secretion is decreased during aging, as is its mediator of action, somatomedin-C (Florini and Regan, 1985). Circulating levels of somatomedin-C apparently result from decreased amounts of growth hormone rather than from any impairment of growth hormone's ability to induce the synthesis of the somatomedins (Florini *et al.*, 1985; Pavlov *et al.*, 1986b).

These events may be of great significance in the development of age-related changes in

several organ systems. That growth hormone—through its mediator, somatomedin C—appears to be responsible for a variety of somatotropic actions, especially the maintenance of muscle mass, has been recently summarized (Rudman, 1985). The notion that aging is associated with a hormone deficiency state (hyposomatotropism) is exciting, since the recent availability of human growth hormone produced by recombinant DNA methodology makes it possible to test the theory. Loss of the muscle mass does contribute in a major way to the functional impairment of aging; prevention or amelioration of this process would seem to be a worthwhile goal. Other postulated effects of growth hormone, such as those on bone, are still more speculative but also intriguing. On the other hand, therapeutic interventions may prove to be difficult, since some evidence is already available indicating that the effectiveness of somatomedin C is impaired at the cellular level during aging (Conover *et al.*, 1985). On a positive note, a recent study shows that administered growth hormone restores the age-related decrease of protein synthesis that occurs in the rat (Sonntag *et al.*, 1985). In this instance, the experimental model does seem appropriate to the situation that occurs in the human.

Catecholamine action

The catecholamine-sensitive adenylate cyclase system is one of the best characterized in the field of endocrinology. A great deal has been learned in the past few years concerning the biochemistry and function of β-receptors and their interaction with the guanine-nucleotide regulatory proteins that in turn interact with the catalytic unit of adenylate cyclase which converts ATP to cyclic AMP (cAMP). Considerable effort has already gone into the study of how aging influences this system. Only one thing is certain: age effects upon this complex system are, themselves, complex (Ebstein *et al.*, 1985; Heinsimer and Lefkowitz, 1985).

The rat fat cell responds to several hormones by accelerating lipolysis. Epinephrine, glucagon, and ACTH are the most potent stimulators of the fat cell's adenylate cyclase. With aging, the cyclase response to glucagon is lost during maturation and can be delayed but not prevented by food restriction, a maneuver which delays a number of other age-related events and extends life in experimental animals. The ACTH response decreases gradually throughout the 24-month lifespan, but the epinephrine response is hardly affected (Fig. 7; Cooper and Gregerman, 1976; Cooper *et al.*, 1977). β-adrenergic receptors are unchanged, as in phosphodiesterase and intracellular cyclic AMP. Nonetheless, maximum lipolytic responsiveness is diminished by about 50% without alteration of the sensitivity to catecholamines (Dax *et al.*, 1981). Thus, even within a single cell type, three distinct patterns of age-related change occur in the hormone-responsive adenylate cyclase system. Furthermore, the mechanism of the change in the catecholamine-related lipolytic response appears to be at some step beyond the adenylate cyclase system. We suspect that there is some alteration of activation of hormone-sensitive triglyceride lipase. Further work in this area may yet dissect out the molecular mechanisms.

In cells other than fat, the age-related catecholamine responses are frequently altered, but the mechanisms appear diverse. For example, in heart (rat), cardiac chronotropic and inotropic action is reduced during aging. Attempts to explain this phenomenon have led to studies of β-adrenergic receptors and adenylate cyclase. The activation of adenylate cyclase by catecholamines is reduced in heart (O'Connor *et al.*, 1981), but receptor number is unaltered. On the other hand, published evidence indicates an age-related

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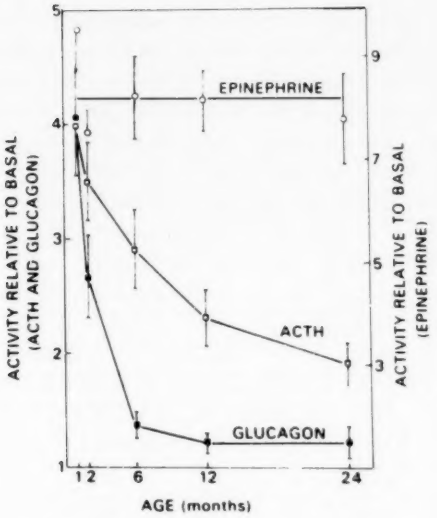

FIG. 7. Relationship of age to the ratio of stimulated to basal activity for hormone-sensitive adenylate cyclase in rat fat cell ghosts. Additions include glucagon, 0.01 and mM (■ —— ■): ACTH, 0.1 mM (□ —— □); and epinephrine, 1 mM (○ —— ○), Results are mean ± SEM for nine experiments. Three different patterns of age-related change in the same type of cell. From Cooper and Gregerman (1976).

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alteration of β-receptor regulation by guanine nucleotides (formation of high affinity state for agonist)—suggesting impaired receptor coupling to the guanine nucleotide regulatory protein—as well as impairment of activation that is non-hormonally mediated through the regulatory protein (Narayanan and Derby, 1982). Both direct quantitation of the regulatory protein and estimates of catalytic unit show age-related decreases of both components (O'Connor *et al.*, 1983). Thus, multiple mechanisms seem to be involved in the decreased cyclase response of aging heart, but simple alteration of receptor number is not one of them.

In lymphocytes (human) diminished activation of adenylate cyclase is also not due to reduced numbers of receptors, but to diminished regulatory protein or catalytic unit activity (Krall *et al.*, 1981). Moreover, the dynamics of the receptor-regulatory protein interaction are affected. β-adrenergic receptors exist in high and low affinity states in which the high affinity state can be converted to low by guanine nucleotides or down-regulated by β-adrenergic agonist (epinephrine, norepinephrine, isoproterenol). A greater proportion of the β-receptors in the lymphocytes obtained from elderly persons in the resting state seem to be in a low affinity form. Down regulation of β-receptors under the influence of increased endogenous catecholamine (norepinephrine) is hardly seen with lymphocytes from the elderly (Feldman *et al.*, 1984). Thus, receptor-cyclase coupling may be impaired and related to the diminished activation of adenylate cyclase. The mechanism of this altered response is not known, but may involve changes of the membranes (see below).

Yet another adenylate cyclase system shows an effect of age on the β-adrenergic receptor-guanine nucleotide regulatory protein interaction. During senescence in rat lung the

β-receptor has been reported not to form a low affinity state under influence of guanine nucleotides (Scarpace and Abrass, 1983). On the other hand, in our laboratory recent attempts to examine this phenomenon in more detail have, to our surprise, yielded no evidence for an age-related alteration of guanine-nucleotide-mediated receptor regulation in heart or lung; regulation in these tissues of old animals seems completely unchanged (Flamer *et al.*, in preparation). An explanation for these discrepant results is not readily apparent, but we do not consider that our results have any greater (or lesser) validity than what has been reported. Rather, the findings suggest to us that some heretofore unappreciated variable is influencing an age-dependent process which affects profoundly the interactions of membrane components of the adenylate cyclase system (see below).

Still another recently described example of an age-related alteration of hormone-sensitive adenylate cyclase has been made with rat kidney cells. Parathyroid hormone increases sodium/calcium exchange in these cells. This response is markedly blunted in cells derived from old animals. The defect appears to be at the level of the receptor or the hormone-receptor coupling to adenylate cyclase and again suggests an alteration of membrane function (Hanai *et al.*, 1986).

Over the past several years our group has had an opportunity to explore an interesting and unusual age-related alteration of hormone-sensitive adenylate cyclase in rat liver. Catecholamine-responsive adenylate cyclase activity increases with age in a selective fashion; the glucagon-responsive enzyme in our strain of rats does not increase (Fig. 8;

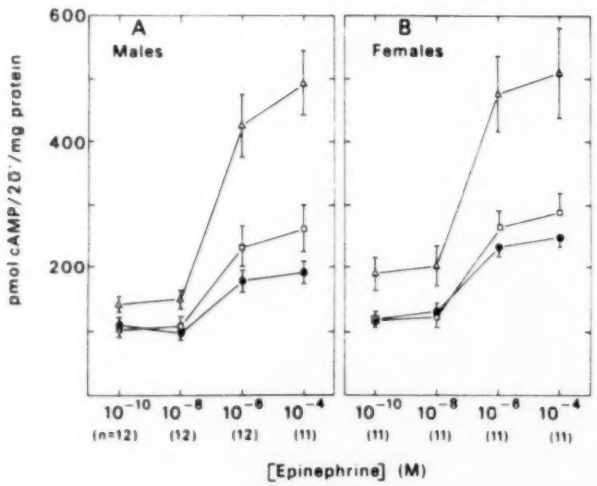

FIG. 8. Epinephrine stimulation of liver homogenate adenylate cyclase from 3-, 12-, and 24-month old rats, males (A) and females (B). Each plotted value represents the mean stimulated activity ± S.E. of n experiments each comparing liver homogenates from three single rats ages 3 (● —— ●), 12 (□ —— □), and 24 (△ —— △) months. For males 12 vs. 24 months, $P < 0.02$, <0.01, <0.005, <0.005 for 10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} M, respectively. For females 12 vs. 24 months, $P < 0.005$, <0.005, <0.001, <0.005. Significance of differences between 3 and 24 months is comparable. Basal values, not shown, are given in Table 11. Note the large increase of catecholamine-sensitive adenylate cyclase in the old animals. From Kalish *et al.* (1977).

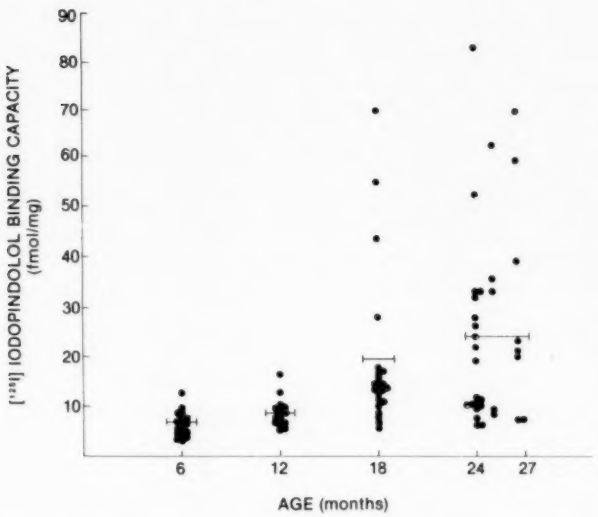

FIG. 9. Increase of β-adrenergic receptors ([^{125}I] iodopindolol binding capacity) in rat liver during aging. From Dax *et al.* (submitted).

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Kalish *et al.*, 1977). (In another strain, a modest increase of glucagon response parallels that of the catecholamine response). From early maturity (2–3 months of age) through about 18 months, β-adrenergic receptors also increase in number and the system seems tightly coupled (Figs. 9 and 10). In old animals (24 months), however, the receptors continue to increase but enzyme activation does not, i.e. the system becomes uncoupled (Dax *et al.*, in press). Several lines of evidence suggest that these changes may be related to physical-chemical alterations of the plasma membrane. The adenylate cyclase activity is a function not only of receptor number, the amount of the cyclase catalytic moiety, and the amount of guanine-nucleotide regulatory (coupling) protein(s), but of interactions of these components within the constraints imposed by the composition of the lipids of the membranes in which they are anchored. Evidence is available that these lipids are altered by aging and can be further altered by nutritional means which in turn affects the activities of several membrane-bound enzymes (Hegner and Platt, 1975; Nokubo, 1985). Age-related changes of biochemical structure of membranes may thus be the basis of a number of age-related alterations of hormone action.

As our understanding of the mechanisms of hormone action has evolved over the past decade or more, those of us interested in gerontology have attempted to determine whether, where, and how age exerts its influences on these processes. It is now obvious and should come as no surprise to us that the age-related changes that have emerged to date are as diverse as the mechanisms of hormone action themselves. We should not be dismayed by such diversity, but if we are to avoid frustration we must be prepared to accept our own diverse roles in what will be a slow unraveling of processes of ever-increasing complexity. In this regard, I was fortunate, in a sense, in my introduction to gerontology. One of my earliest adventures was a study of thyroxine degradation in man in which Dr. Shock was not only a co-author, but a subject as well (Gregerman *et al.*, 1962). Thyroxine degrada-

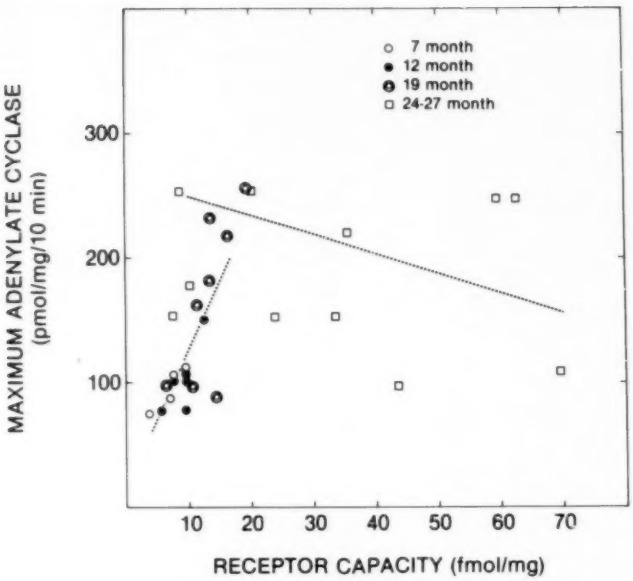

FIG. 10. Relationship between β-adrenergic receptor number and simultaneously determined adenylate cyclase activation in rat liver during aging. Note that in animals up to 19 months the relationship between receptor number (capacity) and maximal activation of adenylate cyclase is close ($r = 0.75$; $P < 0.001$) but that in the oldest animals the positive relationship disappears ($r = -0.35$; $P > 0.1$) indicating uncoupling of the response. This is one of 4 such experiments. From Dax *et al.* (submitted).

tion was decreased by 50%. My attempt to carry on such studies in a rat model so that biochemical mechanisms might be approachable came to a decisive termination when the old rats showed an increase of thyroxine degradation by 50% (Gregerman, 1963). After adjusting to that dead-end, the job was easy; I spent my time examining some of the determinants of thyroxine disposal—from which came some mini-insights on aging (Gregerman and Solomon, 1967).

The next decade and beyond should see—if we are lucky enough to avoid some man-made global disaster of physical or economic genre—further advance in our understanding of these areas of hormone secretion and action. Some others I have not previously mentioned but are certainly here already: calcium as another intracellular messenger, inositides as mediators, hormonal control of gene expression, and others as yet unnamed. Some remain to be discovered. All will have their gerontologic overtones. I hope that there will be enough scientists-administrators of the Shock type to make certain that gerontology continues to build upon these newly emerging areas.

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POSTURAL CHANGES IN CARDIAC VOLUMES IN MEN IN RELATION TO ADULT AGE

RICHARD J. RODEHEFFER*, GARY GERSTENBLITH, ELSIE BEARD, JEROME L. FLEG, LEWIS C. BECKER, MYRON L. WEISFELDT and EDWARD G. LAKATTA

Gerontology Research Center and Cardiology Division, Department of Medicine, Johns Hopkins Medical Institutions, Baltimore, Maryland

Abstract—Cardiac volumes by equilibrium gated cardiac blood pool scans and heart rate were measured in the supine and sitting positions in 64 male volunteer subjects (age 25–80 yrs) who had been rigorously screened to exclude cardiovascular disease. After the upright position was assumed, the average cardiac output of all subjects was unchanged but heart rate increased and stroke volume decreased due to a decrease in end diastolic volume. Neither the supine or sitting cardiac output nor the average postural change in cardiac output, cardiac volumes or heart rate was age-related. While the average cardiac output among the subjects was unaltered with a change in posture, in some individuals it increased slightly while in others it decreased. The postural change in cardiac output among the individuals correlated by linear regression analysis (1) with a change in heart rate only in younger subjects and (2) with a change in stroke volume in all age groups, but the slope of this relationship was greater in older than in younger subjects. The postural change in stroke volume was strongly correlated with a change in end diastolic volume and this relationship did not vary with age. Thus, although the average postural change in cardiac output among healthy subjects is not age-related, a given change in cardiac output with posture in an older individual depends more on a change in stroke volume and less on a heart rate change than in a younger one. This result, like the response to vigorous upright exercise previously demonstrated to occur with aging, indicates a greater reliance in the elderly on the Frank-Starling mechanism than on heart rate for a given change in cardiac output in response to perturbations from the basal supine state.

Key Words: aging, cardiac output, cardiac volumes, posture, autonomic modulation

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INTRODUCTION

THE EFFECT of posture on hemodynamic function has been the subject of numerous investigations, the results of which have been inconsistent. In those studies which have shown the upright cardiac output to decrease below or to remain the same as that in the supine position (Wang *et al.*, 1960; Granath *et al.*, 1961; Bevegard, 1963; Bevegard *et al.*,

*Present Address: Division of Cardiology, Vanderbilt University, Nashville, Tennessee 37232.
Correspondence to: Edward G. Lakatta, M.D., Laboratory of Cardiovascular Science, Gerontology Research Center, 4940 Eastern Avenue, Baltimore, Maryland 21224.
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1963; Granath *et al.*, 1964; Thadani and Parker, 1978; Scholz *et al.*, 1980), stroke volume was uniformly decreased in the sitting position. The precise effect of posture on cardiodynamics, however, could not be determined from these studies since ventricular end-diastolic and end-systolic volumes were not measured. Improvements in equilibrium radionuclide ventriculography allow accurate measurement of end-systolic and end-diastolic ventricular volumes (Pavel *et al.*, 1977; Dehmer *et al.*, 1981; Links *et al.*, 1982). These advances have made it possible to study alterations in absolute ventricular volumes under a variety of hemodynamic stresses including exercise and changes in posture (Poliner *et al.*, 1980; Rodeheffer *et al.*, 1984). In a small number of healthy young subjects it has been demonstrated that cardiac output is not altered by a change from the supine to the sitting position; however, the postural change resulted in a decrease in end-diastolic volume and stroke volume which were compensated for by an increased heart rate (Poliner *et al.*, 1980).

It has been suggested that aging alters the hemodynamic response to a postural change (Granath *et al.*, 1961; Granath *et al.*, 1964; Nixon *et al.*, 1985). Measurements of the effect of posture on absolute left ventricular volumes have not been made across a broad adult age range. In light of the recent demonstration that there is increasing dependence on stroke volume and a decreasing dependence on heart rate as cardiac output increases during exercise in elderly subjects (Rodeheffer *et al.*, 1984), we sought to evaluate whether postural changes in cardiac volumes and heart rate are age related. Specifically, we wished to determine whether a given change in cardiac output with a change in posture would be related to a greater change in stroke volume and a lesser change in heart rate in older vs. younger subjects. Our subjects spanned the adult age range and were carefully screened to exclude overt and occult cardiac disease. They were active community dwellers but not conditioned athletes. This rigorous selection process is important in an attempt to separate the effects of age from those due to differing degrees of physical conditioning or the presence of unsuspected cardiac disease in elderly subjects (Lakatta, 1985). Absolute supine and sitting left ventricular volumes were measured with gated radionuclide angiography (Links *et al.*, 1982).

METHODS

Selection of Study Population

All subjects were volunteer participants in the Baltimore Longitudinal Study on Aging (BLSA). The demographic characteristics of this study group have been described previously (Costa *et al.*, 1981). Participants in the study, although not athletically conditioned, maintain the moderate levels of physical activity required for independent community living and undergo examination every one and a half to two years. All subjects receive a thorough cardiovascular evaluation during each visit, including recording of a history, physical examination, and an electrocardiogram obtained at rest and during maximal treadmill exercise. Stress thallium scintigraphy is performed in individuals over 40 years of age as further screening for occult coronary disease. In the present study BLSA subjects were asked to volunteer for the gated blood pool measurement of cardiac volumes in the resting supine and sitting position if they satisfied the following criteria:

(1) No evidence of cardiac disease on history or physical examination.
(2) A normal resting electrocardiogram.

(3) A normal exercise treadmill test conducted according to a standard protocol (Balke and Ware, 1959) with monitoring of multiple electrographic leads (I, II, III, aVR, aVL, aVF, and V1-V6). In this screening test all subjects exercised to their maximal voluntary level of effort, reached at least 90% of the predicted maximal age-specific heart rate, without J point depression of 1 mm or more with horizontal or downward sloping ST segment for greater than 0.08s in any lead (Minnesota code 4:1) (Blackburn *et al.*, 1960).

(4) In individuals over 40 years of age, a normal maximal exercise thallium test performed according to a previously described technique (Bailey *et al.*, 1977).

(5) An ejection fraction of at least 0.50 at rest and no abnormal wall motion during exercise on the gated blood pool scan.

Gated cardiac blood pool scans

Subjects were studied at midday in the post-absorptive state (Lipsitz *et al.*, 1983). They were placed in the supine position for approximately 20 min before the first gated blood pool scan was obtained. After in vivo labeling of red blood cells with stannous pyrophosphate and technetium-99m, 12 mCi/m2 body surface area (BSA), gated blood pool scans were obtained in an approximately 40 deg left anterior oblique (LAO) position (to best define the interventricular septum) with the subjects in the supine position (Pavel *et al.*, 1977). Images were acquired with a high-sensitivity, parallel-hole collimator attached to a standard Anger camera interfaced with a commercial Nuclear Medicine computer system. Data were acquired on a magnetic disk (64 × 64 matrix, 1.9 × zoom). Acquisition was stopped when 275,000 counts had been collected in a least one of the 14 frames over the cardiac cycle. Heart rate and indirect brachial arterial pressure were obtained in the supine position. The subject was then placed in the sitting position with legs dependent. After a 10 min equilibration period a gated blood pool scan was again obtained in an approximately 40 deg LAO position.

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Left ventricular volumes were determined by a method previously described in detail (Links *et al.*, 1982). Briefly, the end-diastolic count rate was obtained from the 40° LAO image using a manually drawn region of interest corrected for background activity determined from a region of interest drawn lateral and inferior to the left ventricle in the end-systolic frame. Attenuation correction was determined individually for each subject by the use of an anterior static view to measure the attenuation distance from a chest wall marker to the count center of the left ventricle, assuming a linear attenuation coefficient equal to that of water. The blood sample was counted with the same camera/collimator system used for the scintigraphic study and corrected for the time delay between the scintigram and the counting of the blood sample. Left ventricular end diastolic volume (EDV) was obtained from the ratio of the attenuation-corrected end-diastolic count rate from the gated study to the count rate per milliliter from a sample of venous blood drawn at the completion of the study. The EDV determined from the radionuclide image was used to calibrate the left ventricular time-activity curve, yielding left ventricular volume throughout the cardiac cycle. Resting EDV's and end-systolic volumes (ESV's) measured by this method correlate well with corresponding volumes obtained by contrast left ventriculography (EDV by radionuclide imaging = 0.97 angiographic + 3 ml, r = 0.95, SEE = 36 ml; ESV by radionuclide imaging = 1.01 angiographic + 1 ml, r = 0.95, SEE = 33 ml) (Links *et al.*, 1982). Stroke volume was calculated as the difference of EDV and ESV.

The product of stroke volume and heart rate defined cardiac output. Measurements of stroke volume and cardiac output by this method and other related count-based methods have been validated (Dehmer *et al.*, 1981; Vernai *et al.*, 1985).

Data for the first 64 consecutive men who met the entry criteria and had technically satisfactory gated blood pool scans were analyzed and constitute the basis of the present report. The 64 subjects were relatively evenly distributed across the age range of 25 to 80 years. In these subjects height, weight, and body surface area (BSA) measured as (height 0.725) (weight 0.425) (71.84/10000), were not age-related.

RESULTS

Supine and sitting cardiac volumes: Average data

The mean supine and sitting hemodynamic parameters and left ventricular volumes for all subjects are presented in Table 1. In the sitting vs. supine position, heart rate increased, stroke volume, end-diastolic and end-systolic volumes decreased and cardiac output was not significantly different. Table 2 indicates that heart rate, stroke volume, end-diastolic, and end-systolic volumes were not significantly different among the three age groups in either the supine or sitting position. However, there is a slight tendency (p = NS) for stroke volume to increase and for heart rate to decrease with age in the oldest group in both positions. When expressed as a continuous function of age, neither HR nor SV were age related in the supine position: HR = -0.07 [age] + 0.67, $r^2 = 0.07$, $p > 0.43$; SV = 0.36 [age] + 81.30, $r^2 = 0.03$, $p > 0.16$. In the sitting position stroke volume tended to be higher in older subjects but heart rate was not age related: HR = -0.13 [age] + 76.0, r^2 = 03, $p >$ cardiac output among the three groups in either position. The mean postural change in cardiac volumes, heart rate and cardiac output did not differ with age (Table 2c). Figure 1 illustrates the cardiac output in both postures as a function of age expressed as a continuous variable. No age difference is observed.

Table 1. Steady-State Cardiac Volumes and Heart Rate in Supine and Sitting Position

	Supine	*Sitting*	*P*
Heart rate (HR) (beats/min)	63.9 ± 1.2	68.8 ± 1.3	0.001
Stroke volume (SV) (ml)	101.0 ± 3.5	92.6 ± 3.0	0.001
Cardiac output (CO) (L/min)	6.4 ± 0.2	6.3 ± 0.2	NS
Cardiac index (CI) ($L/min/M^2$)	3.3 ± 0.1	3.2 ± 0.1	NS
End-diastolic volume (EDV) (ml)	162.0 ± 5.7	144.2 ± 4.5	0.001
End-systolic volume (ESV) (ml)	61.1 ± 2.5	51.7 ± 2.1	0.001
Ejection fraction (EF)%	62.5 ± 0.7	64.2 ± 0.8	0.002

TABLE 2. STEADY-STATE CARDIAC VOLUMES AND HEART RATE

	Age 25–44 yr. n = 16		Age 45–64 yr. n = 32		Age 65–80 yr. n = 16		Age Effect by Variance Analysis	
	Mn	*SE*	*Mn*	*SE*	*Mn*	*SE*	*F*	*P*
A: Supine Position								
HR	64.6	2.63	64.4	1.88	62.4	1.84	0.31	0.74
SV	93.1	4.98	102.6	5.43	105.8	7.61	0.90	0.41
CO	6.04	0.38	6.53	0.33	6.55	0.48	0.44	0.64
COI	3.07	0.20	3.29	0.15	3.50	0.25	0.92	0.40
EDV	154.3	7.94	167.4	9.12	158.8	10.96	0.49	0.61
ESV	61.4	3.64	65.0	4.04	53.1	4.37	1.91	0.16
B: Sitting Position								
	Mn	*SE*	*Mn*	*SE*	*Mn*	*SE*	*F*	*P*
HR	71.1	2.44	68.6	2.05	67.0	2.47	0.69	0.51
SV	86.5	5.37	92.2	4.66	99.8	5.59	1.21	0.30
CO	6.10	0.39	6.21	0.26	6.50	0.40	0.30	0.74
COI	3.09	0.20	3.14	0.13	3.47	0.20	1.27	0.29
EDV	137.5	7.28	145.8	7.32	147.7	8.03	0.38	0.69
ESV	51.1	2.92	53.7	3.54	48.2	3.70	0.58	0.56
C: Change With Posture								
	Mn	*SE*	*Mn*	*SE*	*Mn*	*SE*	*F*	*P*
HR	6.47	1.35	4.17	1.26	4.60	1.72	0.69	0.51
SV	−6.63	3.93	−10.40	3.38	−6.03	3.30	0.46	0.63
CO	0.054	0.34	−0.316	0.21	−0.053	0.155	0.66	0.52
COI	0.021	0.18	−0.146	0.10	−0.0268	0.08	0.52	0.60
EDV	−16.84	5.33	−21.61	5.07	−11.16	4.91	0.95	0.39
ESV	−10.27	2.10	−11.21	2.48	−4.90	2.38	1.58	0.21

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The Change from Supine to Sitting Position in Individual Subjects

The changes in left ventricular volumes and heart rate from the supine to the sitting position in each subject were first analyzed without respect to age. The mean change in each parameter is illustrated in Fig. 2A. In the sitting position heart rate is increased (4.9 ± 0.8 beats/min), and cardiac volumes are decreased relative to supine values: stroke volume (−8.4 ± 2.1 ml), end-diastolic volume (−17.8 ± 3.1 ml), and end-systolic volume (−9.4 ± 1.5 ml, $p < 0.001$) for each volume. The mean cardiac output, however, is not different in the two postures. Thus with a change from the supine to the sitting posture a decrease in end-diastolic volume results in a decrease in stroke volume but heart rate increases to prevent a fall in cardiac output that would otherwise result from the decline in

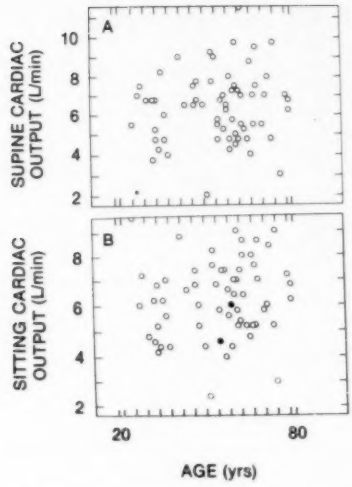

FIG. 1. Cardiac output in the steady state in the supine, Panel A, and upright (sitting) position, Panel B, is not age related. Closed circles indicate more than a single data point.

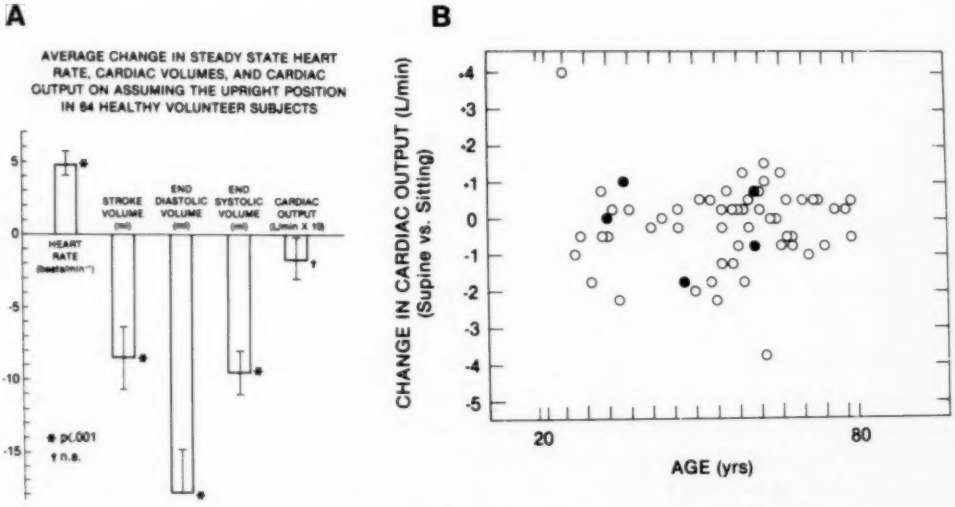
A
AVERAGE CHANGE IN STEADY STATE HEART RATE, CARDIAC VOLUMES, AND CARDIAC OUTPUT ON ASSUMING THE UPRIGHT POSITION IN 64 HEALTHY VOLUNTEER SUBJECTS
HEART RATE
STROKE VOLUME
END DIASTOLIC VOLUME
END SYSTOLIC VOLUME
CARDIAC OUTPUT
* p<.001
† n.s.
B
CHANGE IN CARDIAC OUTPUT (L/min) (Supine vs. Sitting)
AGE (yrs)

FIG. 2. The average (mean ± SEM) difference in heart rate, cardiac volumes, and cardiac output in the supine and upright positions in 64 healthy male subjects. FIG. 2B. The change in cardiac output from the supine to sitting position is not age related. Note, however, that while the *mean* cardiac output does not vary with posutre, in some individuals it increases and in others it decreases. Closed circles indicate more than a single data point.

stroke volume. Fig. 2B shows that the change in cardiac output with posture is not age-related. The large variance about the mean change in cardiac output, however, indicates that after the sitting position was assumed, cardiac output decreased in some individuals while in others it increased slightly. This individual variation in the postural change in cardiac output is illustrated in Fig. 2B.

Using linear regression analysis we determined the relationship in individual subjects between the postural change in cardiac output and the changes in cardiac volumes and heart rate. Whereas a correlation between the change in cardiac output and the change in heart rate was not observed in the overall sample (Fig. 3A), such a relationship was present in the youngest group (Table 3). Table 3 and Fig. 3 show that the postural change in cardiac output in a given subject is highly positively correlated with the change in stroke volume but not with the change in heart rate. This indicates that in those subjects in whom stroke volume decreases less when assuming the upright position, the cardiac output decreases less. Conversely, cardiac output does not decline in those subjects who do not experience a postural decrease in stroke volume. The magnitude of the correlation coefficient indicates that 73–88% of variance in the change in cardiac output with posture among subjects can be accounted for by the change in stroke volume. Table 3 indicates that the slope of this relationship increases with age. Thus, a given change in cardiac output is associated with a greater change in stroke volume in elderly subjects than in younger subjects (compare slope coefficients in Table 3). The postural change in stroke volume is highly correlated with the change in end-diastolic volume (Fig. 3B and Table 3). This dependence of the change in stroke volume on the change in end-diastolic volume did not vary with age (Table 3).

DISCUSSION

The present results, when analyzed without respect to age, demonstrate that the *average* cardiac output measured in a large number of individuals is not significantly altered by changing posture from the supine to the sitting position because an average increase in heart rate offsets an average decrease in stroke volume. Both end-diastolic and end-systolic volumes are smaller in the sitting position, indicating that the reduction of stroke volume with assumption of that position is due to a decrease in end-diastolic volume, rather than to an increase in end-systolic volume (Tables 1, 2 and Fig. 3). Alternatively stated, the postural change in stroke volume is a manifestation of the Frank-Starling mechanism (Tables 1, 2 and Fig. 1).

Our results indicate, however, that while the average cardiac output among subjects does not vary with posture, there is considerable inter-subject variation in the postural effect on cardiac output. In some subjects cardiac output increases slightly with a change from the supine to sitting position while in others it decreases. This indicates that the subtle interplay between the changes in stroke volume and heart rate that determines the postural change in cardiac output with posture varies to some extent among individuals. Whereas the mean postural change in cardiac output is not age related (Fig. 2A), subgroup analyses indicate that a given change in cardiac output in an individual subject is mediated by different mechanisms depending on the subject's age (Table 3). With increasing age, a given change in cardiac output between the two postures in an individual subject exhibits less of a reliance on a change in heart rate and an increasing dependence on a change in stroke volume.

In the steady-state upright position, plasma norepinephrine concentrations in both young

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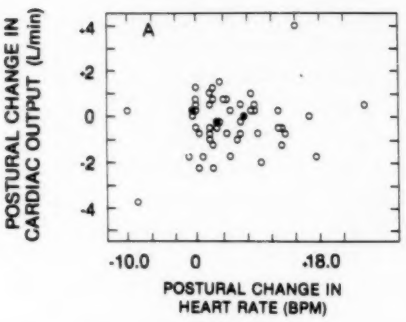

FIG. 3A. Postural changes in steady-state cardiac output vs. changes in steady-state heart rate for all subjects. $\Delta CO = 0.035$ [HR] -0.39, $r^2 = .03$, $p < 0.2$. Sub-group analysis showed, however, that in the youngest subjects the postural change in cardiac output and HR are significantly correlated (see Table 3).

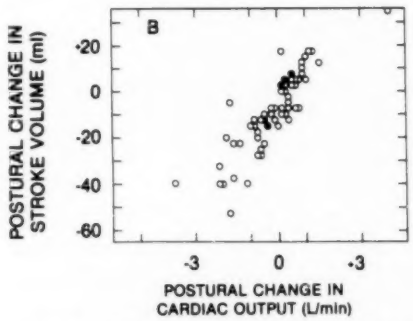

FIG. 3B. Postural changes in steady-state stroke volume vs. changes in steady-state cardiac output among individual subjects without regard to age. $\Delta SV = 12.96$ [ΔCO] -6.32, $r^2 = 0.74$, $p < .001$. The slope of this relation varies with age (see Table 3).

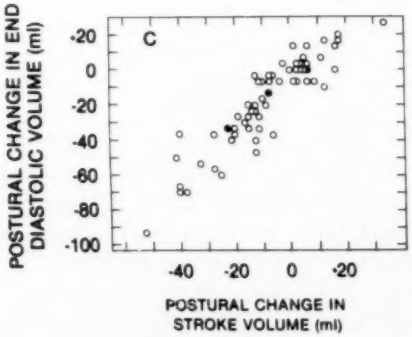

FIG. 3C. Postural changes in steady-state end diastolic volume vs. changes in steady-state stroke volume among individual subjects. $\Delta EDV = 1.35$ [ΔSV] -6.54, $r^2 = 0.83$, $p < 0.0001$. The slope of this relation does not vary with age (see Table 3).

TABLE 3. LINEAR REGRESSION OF POSTURAL CHANGES IN CARDIAC VOLUMES AND HEART RATE ON THREE AGE GROUPS

	Age (Yr)	*Slope Coefficient of Linear Regression Mean ± SE*	r^2	*P*
ΔCO vs. ΔHR	25–44	0.15 ± 0.06	0.30	<0.04
	45–64	0.004 ± 0.04	0.001	<0.93
	65–80	0.005 ± 0.03	0.003	<0.85
ΔSV vs. ΔCO	25–44	10.78 ± 1.06	0.88	<0.001
	45–64	13.72 ± 1.53	0.73	<0.001
	68–80	18.26 ± 2.90	0.74	<0.001
ΔEDV vs. ΔSV	25–44	1.27 ± 0.12	0.88	<0.001
	45–64	1.36 ± 0.12	0.82	<0.001
	65–80	1.34 ± 0.12	0.81	<0.001

and old individuals are higher than in the supine position (Christensen and Bradsborg, 1973; Lake, *et al.*, 1977; Palmer *et al.*, 1978; Robertson *et al.*, 1979; Sussman *et al.*, 1979; Feldman *et al.*, 1983; Feldman *et al.*, 1984); this occurs within 3 min of postural change and remains stable (Sussman *et al.*, 1979). The magnitude of this postural rise in plasma norepinephrine has been reported to be increased in elderly subjects (Ziegler *et al.*, 1976).

Vigorous exercise in the upright position produces a further four to five-fold increase of plasma norepinephrine above the resting level (Robertson *et al.*, 1979; Bartel *et al.*, 1980; Fleg *et al.*, 1985). The plasma levels in older subjects are higher during exercise than in younger subjects (Bartel *et al.*, 1980; Fleg *et al.*, 1985). During upright exercise an age-related increase in end-diastolic volume and stroke volume prevents or reduces the decrease in cardiac output that would occur due to heart rate deficits in elderly subjects (Rodeheffer *et al.*, 1984; Mann *et al.*, 1986). Stated alternatively, a greater reliance on the Frank-Starling mechanism enables the older heart to compensate for a blunted augmentation of heart rate compared to that in younger subjects during exercise.

During exercise in the presence of beta-adrenergic blockade, cardiac output is maintained at the level achieved prior to blockade, despite a blunted chromotropic response, due to compensatory rise in EDV and SV (Renlund *et al.*, 1985). It has been postulated that age dependent changes in exercise hemodynamics, which are remarkably similar to those observed with exercise during beta-adrenergic blockade, may be due to an age-related decline in the responsiveness of the cardiovascular system to beta-adrenergic stimulation (Fleisch *et al.*, 1970; Lakatta *et al.*, 1975; Fleisch *et al.*, 1976; London *et al.*, 1976; Yin *et al.*, 1976; Ziegler *et al.*, 1976; Lake *et al.*, 1977; Kuramato *et al.*, 1978; Palmer *et al.*, 1978; Frolkis *et al.*, 1979; Lakatta, 1979; Strozzi *et al.*, 1979; Vestal *et al.*, 1979; Yin *et al.*, 1979; Bartel *et al.*, 1980; Guarnieri *et al.*, 1980; Lakatta, 1980; Fleisch, 1981; Van Brummelen *et al.*, 1981; Lakatta and Yin, 1982; Feldman *et al.*, 1984; Rodeheffer *et al.*, 1984). Although lymphocyte beta receptor density is unchanged with age, there is evidence that aging is associated with a reduction in receptors of high affinity for beta-adrenergic agonists (Feldman *et al.*, 1983) and this reduction in affinity is accompanied by an age-related increase in plasma norepinephrine in the resting supine and upright positions (Ziegler *et al.*, 1976; Lake *et al.*, 1977; Palmer *et al.*, 1978; Bartel *et al.*, 1980; Feldman *et al.*, 1984). Studies in isolated rat myocardium have shown, in addition, age-related alterations in the

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intracellular response to beta-adrenergic receptor stimulation (Guarnieri *et al.*, 1980; Lakatta and Yin, 1982).

The age differences in cardiac responsiveness to high catecholamine levels demonstrated during vigorous upright exercise are more dramatic than those produced by a less intense adrenergic stimulus, such as assumption of the upright position. Additionally, age related differences in cholinergic modulation of cardiovascular function, should these occur, could also be involved in the age differences observed in the present study. Nevertheless, the present findings are consistent with an age-related decline in cardiac adrenergic responsiveness and suggest that during even the relatively mild adrenergic stimulus of assuming the upright posture, with increasing age a given change in cardiac output is less dependent on a change in heart rate and more dependent on changes in cardiac volumes, specifically more dependent on the Frank-Starling mechanism, as is the case during vigorous exercise (Rodeheffer *et al.*, 1984).

In summary, these findings confirm that in the overall sample, assumption of the upright position results in increased heart rate and decreased left ventricular stroke volume with no change in the mean cardiac output. In a given individual, a change in stroke volume is closely related to a change in end-diastolic volume. The average change in cardiac output with assumption of the upright posture does not vary with age. However, with advancing age the mechanisms that produce a given change in cardiac output in an individual differ: there is a greater dependence on stroke volume in elderly subjects and on heart rate in younger individuals.

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ANATOMIC AND PHYSIOLOGIC AGE CHANGES IN THE KIDNEY

ROBERT D. LINDEMAN

Chief of Staff, VA Medical Center, Professor of Medicine, George Washington University, School of Medicine and Allied Health, Georgetown University School of Medicine, Washington, D.C.

RALPH GOLDMAN

Wadsworth VA Medical Center, and Professor of Medicine/Geriatrics in Residence, University of California at Los Angeles, School of Medicine, Los Angeles, California

Abstract—Cross-sectional studies of renal function in man indicate there is a progressive decline with age after the age of 40 years. The blood vessels, glomeruli, tubules and interstitium are all potential sites of primary involvement in the aging process as well as for renal disease. Regardless of the anatomic structure initially affected, most chronic renal conditions evolve with destruction of the entire nephron. Whether the observed decrease in renal function associated with aging is the result of intervening pathologic processes, e.g. ischemia (vascular obliteration) or infection, or is the result of a more insidious involutional process, it has generated much discussion but few answers. The purpose of this report is to review the descriptive studies documenting the changes in renal morphology and physiology with age and to focus on what is known about the mechanisms involved in these losses of renal substance and function.

Key Words: renal morphology, renal function, age changes, glomerular filtration

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INTRODUCTION

THE INCREASING number and proportion of the aged population, combined with a growing awareness of the clinical significance of age related changes in structure and function, have stimulated the development of a more precise knowledge of these changes. Kidney function can be easily, accurately, precisely, and noninvasively quantified using the clearance techniques developed by Homer Smith. Such measures require only the collection of timed urine samples and blood samples at midpoints of the urine collection periods. These tests have made this organ system a model for studies of aging in man.

CHANGES IN RENAL MORPHOLOGY WITH AGE*

The kidney greatly enlarges from birth to maturity, then, with increasing rapidity, loses mass with age. Roessle and Roulet (1932) found the two kidneys to weigh an average of 50

Correspondence to: Robert D. Lindeman, M.D., Chief of Staff, VA Medical Center, 50 Irving Street, Washington, DC 20422.
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g at birth, 270 g in the third and fourth decades, and to decline to 185 g in the ninth decade. Dunnill and Halley (1973) found the combined volumes of both kidneys to be 20 ml at birth and 250 ml in healthy adults aged 20–25 years. The volume declines in old age and is about 200 ml after the eighth decade. The volume of the cortex decreases much more than does the medulla, and is reduced to less than half of the maximum adult values. Tauchi, Tsuboi, and Sato (1958) present almost identical values, but noted no change in the ratio of cortex to medulla with age. Arataki (1926) reported that the two kidneys of the rat weigh 50 mg at birth and rapidly grow to more than 500 mg in 30 days. The maximum weight, reached at 150 days, is 2.5 g in males and 1.9 g in females. This declines slightly at 350 days, but is significantly reduced to 1.7 g for males and 1.4 g for females at 500 days. The gross loss in renal weight due to aging in both humans and rats appears to be between 20% and 30% of maximum adult values.

The glomeruli

The initial difficulties in counting glomeruli resulted in variable values from 0.56×10^6 (Sappey, 1889) to 5.7×10^6 (Traut, 1923) per human kidney. The high values were obviously too high, but the low values could have been due to the changes of old age or disease. Moore (1931) examined the kidneys of 29 individuals from one to 73 years of age. He concluded that the human kidney contains 800,000 to 1,000,000 glomeruli from birth to forty years, but that occasional values may be as low as 600,000 and as high as 1,200,000 and that there is a senile loss so that by the seventh decade the number may be reduced by one-third to one-half. There was no evidence for post natal nephrogenesis in humans, and each kidney of a normal pair had almost the same number of glomeruli. Dunnill and Halley (1973) counted the glomeruli in 18 individuals whose ages ranged from one day to 73 years, and obtained the same results. The average initial value of one million nephrons per human kidney is now generally accepted.

Dunnill and Halley (1973) also estimated that at birth the filtering surface of human glomeruli was $0.2m^2$. The mean value for all kidneys over 16 years was 1.6 ± 0.7 m^2. The cell to lumen ratios in the glomeruli decreased from 48.5% during the first year to 4.1% after age 16, then stabilized. The cell types were not differentiated in this study, but Wehner (1968) found that the portion of mesangial cells increases significantly from a mean of 6.2% of glomerular volume to 10.4% in old age. The glomeruli comprise 18% of the cortical volume in the newborn, decrease to 8.6% in the adult and then remain constant (Dunnill and Halley, 1973). A number of electron microscopic and PAS studies have revealed reduplication and focal thickening of both glomerular and tubular basement membranes with age (Farquhar *et al.*, 1957; Andrew and Pruett, 1957; Ashworth *et al.*, 1960; Darmady, *et al.*, 1973; McNeill and Dittmer, 1976; Scott, 1964). Recent reports of capillary basement membrane thickening as a possible universal aging phenomenon with obvious physiologic implications lends increased importance to these observations (Kilo *et al.*, 1972).

There is definite evidence that in the rat the number of glomeruli increases the first 100 days of life, remains stable until 350 days, then declines. Arataki (1926) found 10,700 glomeruli at birth, 31,000 at maturity, and 20,000 at 500 days. Glomeruli in the outer portion of the cortex measure 55 μm at birth as compared to those in the juxtamedullary region which measure 68 μm in diameter. Glomerular enlargement reaches 123 μm at 500 days. The diameter of the proximal convoluted tubules remains constant at 40 μm

throughout this period. The number of glomeruli per unit volume of cortex is greater in the female than in the male. The number of glomeruli per cubic millimeter cortex decrease from 1000 at birth to 70 at 100 days and 60 at 500 days, and is due to the increase in glomerular size and to the maturation of the renal tubules. Thus the glomeruli make up 11% of the cortical volume at birth, 3.5% from 30 days to 150 days, increasing again slowly to 5.2% in males and 6.4% in females at 500 days. A similar, but less marked, increase in average rat glomerular diameter was found by Moore and Hellman (1930).

Several investigators (Tauchi *et al.*, 1971; Goyal, 1982) have reported that a decrease in the number of parenchymal cells and increase in the size of cells also are the main characteristics of aging changes in man. Both glomerular and tubular cells decrease in number as do the number of glomerular tufts per unit area. The size of malpighian corpuscles, glomerular tufts, nuclei of glomerular cells and nuclei of tubular cells all increased significantly in older kidneys.

Despite the extensive nephron obsolescence which is known from other evidence, there seems to be insufficient residual scarring to account for the loss. This could well be because the process is so diffuse and very slowly continuous over a long period of time (Tauchi *et al.*, 1958; Darmady *et al.*, 1973; Moore and Hellman, 1930; Oliver, 1939). Glomeruli begin to degenerate in the pelvic and periarcuate region as early as the seventh intrauterine month, there is scattered atrophy of cortical glomeruli after the ninth intrauterine month, and by the seventh year degenerated juxtamedullary glomeruli can be seen (Ljungvist, 1963). Tauchi *et al.* (1958) found the number of glomerular scars did not exceed 10%. McLachlan *et al.* (1977) reported similar values in men, although women showed no increase with age, but Kaplan *et al.* (1975) observed that the average slightly exceeded 10% after age 50, with a 95% confidence range, and increased to 27% by age 70. Sworn and Fox (1972) found a significant correlation between age and number of abnormal glomeruli by histologic criteria. There was a decrease from the equivalent of 95% normal glomeruli under age 40 years to 63% at 90 years. All individuals possess at least 50% normal glomeruli until age 80. None of these authors attempted to relate the fractional number of scarred or obsolescent glomeruli counted to the total number of original nephrons, but only to the number of residual nephrons at various ages.

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The age-related chronic glomerular lesions observed in rats, hamsters and mice are discussed in more detail in the section on glomerular permeability. With age, there is a thickening of the glomerular basement membrane due to accumulation of basement membrane-like material on the epithelial side of the lamina densa (Yagihashi and Kaseda, 1978; Yagihashi, 1978). There is a thinning of the lamina rarae interna and externa. With age, epithelial cells show less numerous cytoplasmic organelles, many myelinated bodies suggesting autophagosomes, and electron dense, "whorl-formed" secretory bodies. There also was increasing mesangial matrix with age. Finally, Kalant, *et al.* (1977) have shown an increase in the collagen content of whole glomeruli and basement membrane with age.

The tubules

Oliver (1939) reported the results of his own microdissections in his pioneer review of the aging kidney. He found little correlation between the size of the glomerulus and the size of the tubule, and that the tubule does not inevitably degenerate after loss of its glomerulus. The individual tubules become reduced in volume when they do degenerate, and are replaced by connective tissue with little evidence of inflammatory or round cell in-

filtrate, suggesting a response to gradually increasing ischemia. Tubular hypertrophy occurs in the remaining nephrons, and is limited almost entirely to the proximal convoluted tubule, which may become as much as 12 times the normal size. This change appears to result from both hypertrophy and hyperplasia of tubule cells. The glomerulus may be normal, enlarged, atrophied, or destroyed. Tauchi *et al.* (1958) showed, in contrast, that in the senile nephron there was a decrease in the volume of the glomerulus, a dilatation of the tubular lumen, and a decrease in the number of cells of both, although the glomerular cells were atrophic and the tubular cells hypertrophic.

The relative decrease in the glomerular component of the cortex which begins immediately after birth is due mainly to the increase in the length and volume of the proximal convoluted tubule (Darmady *et al.*, 1973) which continues to maturity, then gradually declines with age. The mean proximal tubule length decreases from 19.4mm (maturity) to 12.5mm (age 70–80 years), and volume decreases from $0.76mm^3$ (maturity) to $0.52mm^3$ (age 70–80 years). The ratio of the glomerular surface area to the proximal tubular volume decreases from 29.4 at term to 11.8 at four months, 7.78 at one year, and stabilizes at 3.2 throughout maturity and into old age. Darmady *et al.* (1973) also failed to find evidence in their microdissection studies, for the aglomerular nephrons reported by Oliver (1939), nor of compensatory hypertrophy or hyperplasia in residual nephrons. Dunnill and Halley (1973) found that the surface area of cortical tubular basement membrane increased from $0.8m^2$ at birth to a mean of $8.7 \pm 2.3m^2$ after the age of 16 years, and was linearly related to renal cortical volume. The evidence suggests that not only does the number of nephrons decrease with age, but that there is also a decrease in the size of those that remain.

The tubular basement membrane, like that of the glomerulus, thickens with age. Scott (1964) found that the basement membranes of rats become irregularly thickened independent of dietary variations. The thickening is accompanied by a regular irregularity of the adjacent portion of the tubule cells, which protrude into the membrane.

Distal tubular diverticuli have been described (Darmady *et al.*, 1973; Peter, 1972; Loomis, 1936; Baert and Steg, 1972) which vary from side pouches to pedunculated sacs, and are in the region of attachment to the macula densa (Darmady, 1973). They connect to the tubular lumen and have been noted to contain detritus and bacteria and may be of clinical significance. The diverticula are rare until age 16 to 18, but then the number increases linearly with age until age 90 when they average 300 per 100 nephrons (Darmady, 1973).

The renal vasculature

The major renal arteries may decrease in diameter with age (Loomis, 1936). However, there is little direct evidence that change in these arteries causes the renal anatomic and physiologic decrements noted. The preoperative evaluation of kidney donors in several studies included arteriography and excluded overt large vessel disease and evidence of infarcts (Ogden, 1967; Boner *et al.*, 1973; Hollenberg *et al.*, 1974; Slack and Wilson, 1976), yet the decrements in renal function paralleled studies of subjects less rigorously selected. The diffuse distribution of atrophic glomeruli and the universal changes in the remainder implies that if the large vessels are involved, an adequate change should be seen in the main renal artery or that equal changes should be noted in each of its major segmental branches. On the other hand, the large vessels decrease in size in response to the renal

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atrophy of parenchymal renal disease, and the reduction in large vessel size may not be the primary event. The segmental arteries give rise to the interlobar arteries, which, with the arcuate arteries, are described as medium-sized. The interlobular arteries are small vessels, leading to the afferent arterioles.

Kidneys radiographed after injection with radio-opaque media at postmortem examination show that the normal renal vessels taper gradually from hilum to cortex and the arcuate arteries curve smoothly (Ljungvist and Lagergren, 1962; Davidson *et al.*, 1969). The age progression is irregular, but abnormalities appear after age 40, and all kidneys show some degree of abnormality after age 80. The failure of the vessels to taper is the most frequent finding. The changes in the arcuate arteries show the best correlation with age; they become angulated and there is notching at the bifurcations. The interlobar arteries may also become tortuous, spiralling, and have luminal irregularities. Polar areas of ischemia are often seen.

Muscular arteries have been reported to undergo deposition of collagen between the intima and the internal elastic lamina, and replacement of muscular cells with collagen. The changes are not usually associated with any decrease in luminal size (Tauchi *et al.*, 1958; Darmady *et al.*, 1973; Oliver, 1939, Williams and Harrison, 1937; Yamaguchi *et al.*, 1969). There is general agreement that changes occur slowly in the larger arteries, but develop later and more rapidly in the smaller vessels. Hypertension is unquestionably associated with an acceleration and intensification of these changes. Tauchi *et al.* (1971) compared a large number of autopsy tissues of Japanese and Caucasian males, and found that sclerosis appeared earlier and was more severe in the Japanese than in Caucasian. Jackson *et al.* (1968) have challenged the evidence for splitting of the elastic lamina and have produced histochemical evidence that what appears to be reduplicated elastin is actually collagen. There is no question that whatever their origin the age-related changes result in diminished elasticity.

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Normal arterioles show little age change. Mild hyalinization is the only arteriolar lesion commonly associated with aging (Tauchi *et al.*, 1958; Ogden, 1967; Ljungvist and Lagergren, 1962; Williams and Harrison, 1937; Yamaguchi *et al.*, 1968). The arteriolar changes of intimal proliferation, medial hypertrophy and necrosis are relatively rare in the absence of hypertension. Moritz and Oldt (1937), Williams and Harrison (1937) and Bell (1946) all found lesions in only 15% of normotensive individuals over the 50, but these observers found arteriolar changes in 58% to 97% of kidneys when hypertension was present. The results of Yamaguchi *et al.* (1969) are illustrative. The ratios of lumen diameter to wall thickness in the small arteries and the arterioles were unaffected by age in the absence of hypertension. When hypertension was present, the ratios were greatly reduced, and most markedly in the younger individuals (Table 1).

MacCallum (1939) and later McManus and Lupton (1960) have presented a detailed description of the course of ischemic obsolescence of glomeruli. The first stage is a progressive collapse of the glomerular tuft, with wrinkling of the basement membranes, followed by a simplification and reduction in the vascular channels. Hyaline is deposited within both the residual glomerular tuft and in the space of Bowman's capsule. Identifiable structures disappear rapidly. The obsolete glomerulus may be reabsorbed and disappear entirely; reabsorption is suggested because of the scantiness of the cellular response and residual scar. This process can leave a single vessel in place of the glomerular capillary, thus producing a shunt between the afferent and efferent arterioles, the

TABLE 1. THE EFFECTS OF AGE AND HYPERTENSION ON THE LUMEN DIAMETER: WALL THICKNESS RATIOS OF SMALL RENAL ARTERIES AND ARTERIOLES (YAMA GUCHI *et. al.*, 1968).

	Arteries		*Arterioles*	
	Young	*Old*	*Young*	*Old*
Normal	3.91	3.84	2.52	2.66
Hypertensive	2.26	2.52	1.51	1.97

arteriolae rectae of Ludwig. Identification of this process and demonstration of arteriolar shunts does much to satisfy the objection that regression of glomeruli must leave tangible evidence of their former presence.

Ljungvist (1962, 1963, 1964) has performed extensive microangiographic studies which confirm the presence and development of the shunts, and relates them to the life cycle of the kidney. Pelvic and juxtamedullary glomeruli degenerate to form shunts as early as the seventh intrauterine month (Wehner, 1968). Scattered cortical glomeruli begin to atrophy after the ninth fetal month, and juxtamedullary glomeruli external to the arcuate vessels

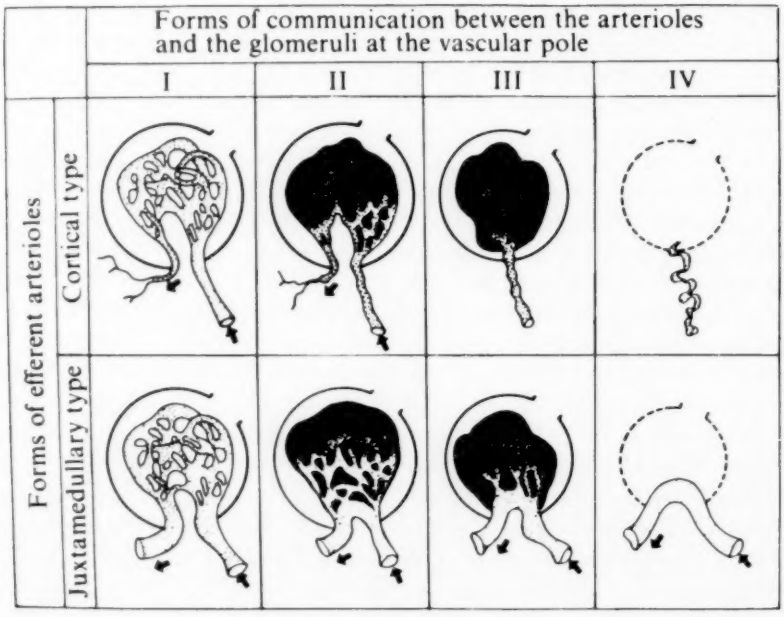

FIG. 1. Structure and classification of the arteriole-glomerular units are classified into two basic types, cortical and juxtamedullary, depending on the form of the efferent arterioles. Cortical types were arranged in various stages of degeneration terminating in complete atrophy of the arteriole-glomerular units. The juxtamedullary type departed from this basic pattern to become continuous where the afferent and efferent arterioles formed a direct connection. (Reprinted by permission from Takazakura *et al. Kidney Int.* 2,224–230, 1972).

after the seventh year of life. More careful examination revealed that in the cortical area atrophy of a glomerulus resulted in the abrupt termination of the arteriole (Fig. 1). Surprisingly, these arterioles remain patent, despite their blind ends, and become more numerous with age, but are still relatively uncommon. In the juxtamedullary region the afferent and efferent arterioles are of the same caliber, unlike the outer cortex, where the efferent arteriole is considerably smaller. In this region there is increasing evidence of aglomerular vessels leading to the vasa rectae, but a majority of the glomeruli still remain intact. A smaller number of altered juxtamedullary glomeruli can be seen which are only partially filled.

Takazakura *et al.* (1972) have confirmed all of these observations. They demonstrated that shunting was present in 9% of the juxtamedullary glomeruli at birth, and that there was a linear increase with age which reached 100% during the ninth decade (Fig. 1). In disease states, including hypertension, the amount of shunting was increased independent of age. Further support is found in the observation of Reynes *et al.* (1968). Reference to Fig. 1 shows glomeruli replacement, rather than the ischemic collapse described by McManus and Lupton (1960).

Payan and Gilbert (1967) found a micronodular phlebosclerosis, consisting of a fibromuscular nodule involving the media of a venule, seen on low power microscopy to be protruding into the vascular lumen, and increasing in number of with age. These nodules are most common in the adrenal, less frequent in the renal cortex, and were not seen in other organs. They also report observation of interrupted, eccentric longitudinal muscle fibers in the renal veins (Payan and Gilbert, 1967). These bundles are not present at birth and rarely in childhood, but are found in 20–33% of adults and 40% of individuals over 60 years. These findings are not associated with hypertension and their significance is not known. They were not found in the renal veins of rats at any age.

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Although it is widely assumed that the age-related changes in the kidney are vascular in origin, the evidence is not conclusive. The major vascular changes are in the small arteries and in the glomeruli. Although the arteries and arterioles show changes, significant luminal narrowing is rare. Those glomeruli which can be observed to involute often retain patent afferent arterioles (cortical glomeruli), or form shunts which become arterioli rectae verae (juxtamedullary glomeruli). The major changes thus could occur at the capillary level, and the glomerular regression could be initiated there by either vascular or tissue factors. The evidence for thickening of the basement membrane may be a significant clue to the mechanism.

The interstitial tissue

Although the cortex shows very little increase in connective tissue with age, there is a significant increase in the medulla, particularly in the pyramids (Darmady *et al.*, 1973; Helpop, 1933; Keresztury and Megyeri, 1962). Dunnill and Halley (1973) estimated the volume of the cortical interstitial tissue to be 11.7% ± 5.5% for individuals below 36 years and 15.5% ± 3.0% for those older. This difference was not statistically significant. The cellular population of the medulla is gradually replaced by intercellular materials, especially after 50 years of age, and this is so extensive that Keresztury and Megyeri (1962) use the term medullary sclerosis. The tissue is vascularized, so they postulate that the process is due to reduced blood supply rather than to vascular occlusion. The presence of finely dispersed sudanophilic fat which parallels the amount of sclerosis is identified by

histochemical staining (Peter, 1927; Keresztury and Megyeri, 1962; Prym, 1909). Finely dispersed deposits of calcium were found in 32.3% of cases under 50 years and 41.8% of those over 50 years of age, but do not appear to be related to the sclerotic process.

Biochemical Changes

The renal content of soluble collagen, insoluble collagen and elastin in 1,8, and 24-month old rats was examined by McGavack and Kao (1960) who found no significant differences. In contrast, Schaub (1963) found the labile collagen to be 30% of the total at birth, 4% at 10 months, and constant thereafter. The total amount of collagen, measured as hydroxyproline, increased from 304 to 857 mg/100g defatted dry weight. Ber *et al.* (1969) determined the concentration of acid mucopolysaccharides (AMPS), expressed as uronic acid, and of hydroxyproline in dry, defatted kidney tissue of male rats as a function of age. They found that the uronic acid increased threefold from birth to eight months, then decreased by one-third to 18 months. Hydroxyproline, representing collagen, increased continuously at all ages, and was over two and one-half times as concentrated at 18 months as at birth.

Inoue *et al.* (1970) studied 20 human kidneys obtained at postmortem from cadavers ranging from 1 to 84 years. They found no changes in the cortical concentration of AMPS, but there was a three-fold increase from birth to age 40 in the medulla, followed by a modest subsequent decline, similar to the pattern found in the rat. Later, they confirmed the rise in AMPS in the papilla to age 70 (Inoue *et al.*, 1973), and noted a decrease in the medullary water content after age 50, so that there is an age-related change in the ratio AMPS: water. Murata and Horuchi (1978) quantified the various acidic glycosaminoglyans (AGAG) in human kidney as they changed with age. Heparan sulfate accounted for 43 to 74% of the total AGAG and this percentage increased with age. The proportion of hyaluronic acid increased with age after age 21 years; the percent of dermatan sulfate did not change with age. The ratio of 4-type chondroitin sulfate to 6-type chondroitin sulfate decreased with advancing age.

Smalley (1980) studied the amino acid concentrations in whole human glomerular basement membrane preparations. He reported decreases in threonine, methionine, isoleucine, leucine, tyrosine, phenylalanine, hydroxylysine and histidine. Decreases in the ratios of 3-hydroxyproline to 4-hydroxyproline and hydroxylysine to lysine were interpreted to represent a reduction in the collagenous protein content in the GBM with age.

Another frequently observed phenomenon in the composition of basement membrane is the increase in hydroxylation of proline and lysine with age. The increase in the concentrations of 3-and 4-hydroxyproline and the ratio of 3-hydroxyproline to 4-hydroxyproline in kidney cortex of man appears to be a developmental rather than an aging change (Langeveld *et al.*, 1981; Deyl *et al.*, 1978).

Finally, Christensen and Madsen (1978) showed that although the volume of autophagic vacuoles (lysosomes) in the proximal tubules increases with aging, there is decreased protein catabolism which could be due either to decreased synthesis of lysosomal enzymes or due to inhibitory substances in the lysosomes.

CHANGES IN RENAL FUNCTION WITH AGE

The observation has been made repeatedly that the decreases in specific renal functions with age, as with various types of renal pathology, parallel the decrease in glomerular

filtration rate. Bricker *et al.* (1960) at first speculated this meant that diseased nephrons did not contribute importantly to the development of the final urine (intact nephron hypothesis). Later, after Biber *et al.* (1968) showed, using micropuncture techniques, that diseased and damaged nephrons can contribute significantly to urine formation, Bricker (1969) revised his hypothesis to incorporate these observations suggesting that healthy nephrons had to compensate for glomerulotubular imbalances that develop in damaged nephrons if the individual was to survive. Except in conditions where specific tubular dysfunction develops, e.g. renal tubular acidosis and nephrogenic diabetes insipidus, changes in other measures of renal function tend to parallel changes in glomerular filtration rate.

Glomerular filtration rate

A number of studies have been published showing an age-related decline in renal function after the age of 30 years (Beck and Vignow, 1966; Davies and Shock, 1950; Hansen *et al.*, 1970; Lewis and Alving, 1938; Muether *et al.*, 1967; Pelz *et al.*, 1965; Siersbaek-Nielsen *et al.*, 1971; Stewart, 1959; Van Pilsum, 1958; Wesson, 1969).

Lewis and Alving (1938) were the first to observe this decrease which they documented by quantifying urea clearances. Davies and Shock (1950) were the first to determine inulin clearances, generally accepted as the most accurate and precise measure of glomerular filtration rate, in aging individuals free of cardiovascular, renal and acute illnesses by history and examination. Seventy male subjects showed a decline in GFR between ages 40 and 80 years which averaged approximately one cc per minute per year of age. Wesson (1969) incorporated these findings with data collected from 36 additional studies where individual inulin clearances and age were recorded, and found an accelerating decrease in GFR with increasing age in both males and females (Fig. 2). The rate of decline was more rapid in the males.

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Most aging studies have been cross-sectional, comparing measurements made on subjects of various ages, and attributing the differences observed to the effect of age. However, results of these studies do not necessarily reflect true age changes as the elderly subjects represent highly selected survivors who may not be representative of the initial cohort, and furthermore, the physiological characteristics of cohorts may change appreciably with calendar time.

The limitations of a cross-sectional study might be avoided by a longitudinal study (serial measurements) in the same individuals over a period of years. Even longitudinal studies have limitations as subtle changes in laboratory techniques may develop over a period of time and increasing familiarity (decreased stress) with physiological tests can produce changes which may be difficult to separate from age-related changes. The difficulties encountered in the interpretation of data collected from cross-sectional and longitudinal studies are discussed in more detail in a review by Rowe (1977).

Rowe *et al.* (1976) reported a ten-year analysis of the first large scale longitudinal study of renal function. Serial true creatinine clearances were obtained on 884 community dwelling volunteers at 12 to 18 month intervals at the Gerontology Research Center in Baltimore. There was an accelerating decline in creatinine clearances similar to that observed in cross-sectional studies, indicating that selective mortality and cohort differences had no significant impact on the cross-sectional results.

Although mean creatinine clearance rates fell from 140 cc/min/1.73 m^2 at age 25–34 years to 97 cc/min/1.73 m^2 at age 75–84 years, serum creatinine concentrations rose in-

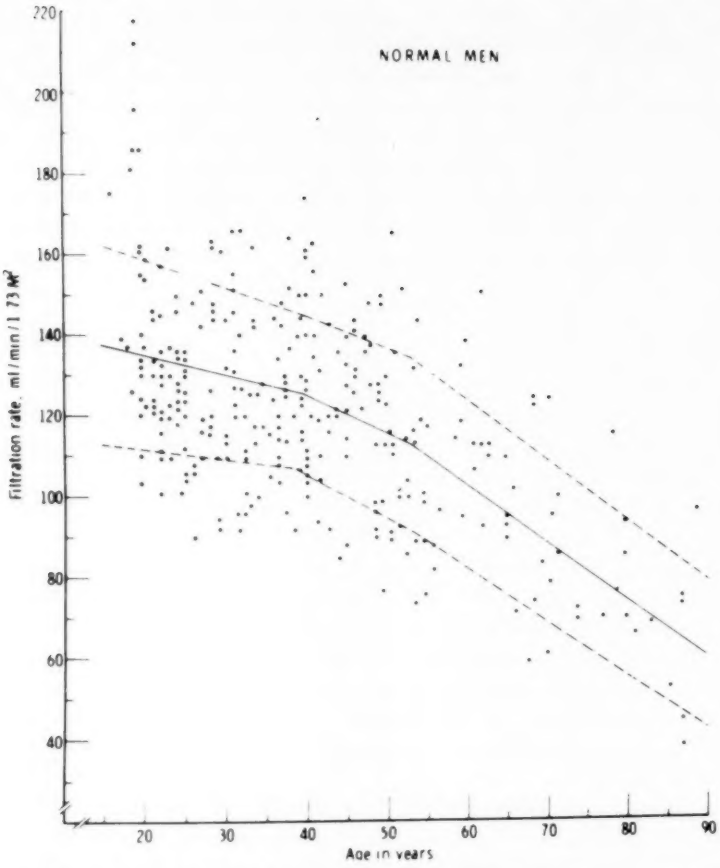

FIG. 2. Glomerular filtration rates (inulin clearances) per 1.73 M^2 in norman men vs. age in 38 studies. The solid and broken lines represent one standard deviation. (Reprinted by permission from Wesson, Physiology of the Human Kidney, Grune and Stratton, New York, 1969).

significantly from 0.81 and 0.84 mg/100 ml. The proportionate decrease in creatinine production is a reflection of the decrease in body muscle mass that occurs with age. The practical implication of these observations is that serum creatinine concentrations in older patients must be interpreted with this in mind when they are used to determine or modify dosages of drugs cleared by the kidneys, e.g. digoxin and the aminoglycoside antibiotics.

The most recent observations to be published from this longitudinal study (Lindeman *et al.*, 1984; Lindeman *et al.*, 1985) indicate that there are a number of individuals, even in the older age categories, who have shown no decrease in their creatinine clearances when studied serially at 12 to 18 month intervals on at least 5 separate occasions. Linear regressions of creatinine clearances on age were calculated for 446 volunteers followed between 1958 and 1981. The mean decrease in creatinine clearance was 0.75 ml/min/year. Figure 3 shows the individual regression equations for creatinine clearances vs. age (time in years) for 254 subjects free of renal and urinary tract disease and untreated with diuretics and

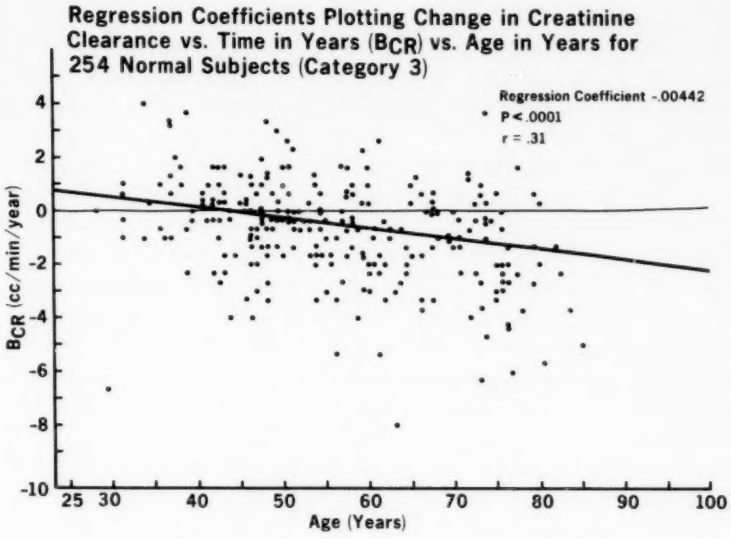

FIG. 3. Regression coefficients plotting change in creatinine clearance vs. time in years (B_{cr}) for 254 individual "normal" subjects (free of renal and urinary tract disease and untreated with diuretics and antihypertensive agents) vs. age in years. Subjects with positive B_{cr}'s had slopes which showed an increase in creatinine clearance with age. (Reprinted by permission from the American Geriatrics Society, Longitudinal studies on the rate of decline in Renal function with Age, by Lindeman *et al.*, *J. Am. Geriat. Soc.* 33, 278–285, 1985).

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antihypertensive agents ("normal" subjects). The slopes of the creatinine clearances vs. time fell into a normal (Gaussian) distribution around this mean. One third of all subjects followed had no absolute decrease in renal function (positive slope of creatinine clearance vs. time). There was a small group of patients who showed a statistically significant increase ($p < 0.05$) in creatinine clearance with age. These observations may have important implications in understanding the pathophysiology of the decrease in renal functions observed with age and will be discussed later.

Renal blood and plasma flow and filtration fraction

The quantity of blood (or plasma) perfusing the kidney, estimated by measuring paraaminohipuric acid (PAH) clearances, decreases with age (Davies and Shock, 1950; Wesson, 1969; McDonald *et al.*, 1951; Lindeman *et al.* 1966; Adler *et al.* 1968). Miller, McDonald and Shock (1961), confirming the work of Bradley (1947), showed the extraction ratios at low arterial PAH concentrations were approximately 92% and were not influenced by age. The PAH clearance in men fell from a mean of 649 cc/min during the fourth decade to a mean of 289 cc/min during the ninth decade (Davies and Shock, 1950), a decrease of 6.44 cc/min per year, which was proportionately slightly greater than the decrease in inulin clearance with age.

This more rapid fall in RPF is reflected by an increase in the filtration fraction, calculated by dividing glomerular filtration rate (inulin clearance) by effective renal plasma flow (PAH clearance). Davies and Shock (1950) showed an increase from 0.19 to 0.23

over the age range studied; this increase has been confirmed by others (Wesson, 1969; McDonald *et al.* 1951; Lindeman *et al.* 1966). This is not totally predictable. One might anticipate that with formation of shunts as glomeruli become obsolescent in the corticomedullary portion of the kidney, the glomerular filtration rate might decrease more rapidly than the renal blood flow.

The decrease in renal blood flow with age without a decrease in blood pressure is indicative of either vascular obliteration due to intraluminal pathology (sclerosis, atheromata) or to an increase in renal vascular resistance due to vasoconstriction. Since renal blood flow can be increased transiently by administration of pyrogen in both young and old subjects (McDonald *et al.*, 1951), a reversible or vasoconstrictive component must be implicated in the regulation of the renal circulation in both age groups. Administration of pyrogen produces a greater vasodilatation in the arteriolar system of the kidney in the older subject, suggesting that a greater resting vasoconstriction exists. Administration of pyrogen to aminopyrine-treated subjects failed to alter GFR but produced a marked increase in ERPF; furthermore, the age-related difference in filtration fractions disappeared, being 0.115, 0.123 and 0.122 in young, middle-aged, and old subjects respectively, compared to 0.189, 0.206, and 0.232 in the control state (McDonald *et al.*, 1951).

The peripheral vascular resistance, determined by dividing mean arterial pressure by cardiac output, increases with age (Landowne *et al.*, 1955). Furthermore there is a decrease in the fraction of the total cardiac output received by the kidney with age (Landowne and Stanley, 1960; Bender, 1965). These findings along with the observation that the filtration fraction increases with age and this increase can be abolished with pyrogen infusion, suggest that the intrarenal resistance in older persons, and that the relatively greater decrease in renal blood flow with age, is a physiologic rather than an anatomic phenomenon with both afferent and efferent arterioles involved in the pressure regulation. One might speculate that since cardiac output decreases with age, the greater renal vasoconstriction in older subjects might be an attempt to maintain better the blood supply to other vital organ systems.

Hollenberg *et al.* (1974), using xenon washout techniques, found that the perfusion of outer cortical nephrons fell more with age than did perfusion of the corticomedullary nephrons. Whether this selective decrease in cortical nephron perfusion was due to sclerotic changes in the small arcuate arterioles or represented a selective vascoconstriction of the peripheral vasculature was investigated. Acetylcholine increased renal blood flow in both young and old subjects but the effect was more striking in the younger subjects. In contrast, the vasoconstrictor response to angiotensin was similar in the young and old subjects. These studies suggest that the renal vasculature in the aged patient is in a relatively greater state of baseline vasodilatation compared to the renal vasculature of a young person in contradiction to the data published by McDonald, Solomon and Shock (1951) which suggest a greater state of resting vasoconstriction in the older patients.

Because the rapid, or cortical, component of flood flow decreased more rapidly than did mean flow rate, it was assumed that cortical nephrons are more severely affected by age than are juxtamedullary nephrons. The authors argue that the juxtamedullary nephrons have a higher filtration fraction than do cortical nephrons so that a selective loss of the latter would explain the increase in the mean filtration fraction observed with age. An alternative but less likely explanation for the increase in filtration fraction observed with age would be that there is a disproportionately greater vasoconstriction of the ef-

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ferent arteriole as opposed to the afferent arteriole in older subjects, thereby increasing the filtration pressure in the glomerular capillary beds.

An additional finding of interest was that modification of sodium intake affected renal hemodynamics differently in young and old subjects. In young subjects, renal blood flow varied directly with salt intake while in older subjects, renal blood flow was unaltered by variations in salt intake.

These two studies are important as they deal with the anatomic versus the physiologic mechanism for the reduction in blood flow. If the anatomic lesions are primary, then they must precede the loss of nephrons. If the physiologic mechanisms are primary, then measures directed at this defect might reduce the rate of nephron loss. The effects upon cardiovascular dynamics of these two mechanisms by which reduced renal blood flow can be achieved obviously merit further study.

Maximum tubular transport capacity

The tubular maximum for diodrast or PAH secretory transport decreases with age at a rate nearly parallel to the decrease in inulin clearance (Davies and Shock, 1950; Miller *et al.*, 1951; Watkin and Shock, 1955). The tubular maximum for glucose reabsorption also decreases with age at a rate closely paralleling the decrease in inulin clearance (Miller *et al.*, 1952).

Although the reduction in the secretory and resorptive tubular maximums with age could be explained by a progressive loss of functional nephrons, animal experiments have shown fewer energy producing mitochondria (Barrows *et al.*, 1960), lower enzyme concentrations (Barrows *et al.*, 1960; Wilson and Franks, 1971; Burich, 1975), lower concentrations of total or sodium-potassium activated ATPase activity (Beauchene *et al.*, 1965), decreased sodium extrusion and oxygen consumption (Proverbio *et al.*, 1985), and decreased tubular transport capacity in the tubular cells (Barrows *et al.*, 1960) in old compared to young kidneys. Thus aging not only results in a reduction in the nephron population but produces changes in the basic biochemistry of the tubular cell even though no changes in tubular function can be detected using available techniques in human studies.

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Concentrating and diluting ability

A decrease in concentrating ability with age now has been well documented in men (Lewis and Alving, 1938; Lindeman *et al.*, 1966; Lindeman *et al.*, 1960; Miller and Shock, 1953; Dontas *et al.*, 1972; Rowe *et al.*, 1976). The most recent study (Rowe *et al.*, 1976) in active, community dwelling men over a wide range of ages has shown that 12 h of water deprivation increased urine osmolality to a mean of 1109 mOsm/L in young subjects (mean age 33 years), 1051 mOsm/L in middle aged subjects (mean age 49 years), and 882 mOsm/L in old subjects (mean age 68 years). Attempts to relate the decrease in concentrating ability to the more rapid decrease in GFR than solute load, presumably resulting in an increased solute load in surviving functional nephrons (an osmotic diuresis would impair the ability to concentrate the urine), have not been successful (Lindeman *et al.*, 1960; Rowe *et al.*, 1976). Rowe *et al.* (1976) felt that the development of a continuity between juxtamedullary afferent and efferent arterioles would increase medullary blood flow. The observations by Hollenberg *et al.* (1974) showing preferential perfusion of juxtamedullary nephrons in older subjects supports this contention. McLachlan (1978) also

raises the possibility that there might be an earlier degeneration of the long-looped juxtamedullary glomeruli with establishment of the continuity between afferent and efferent arterioles and that this selective obliteration of these nephrons before the superficial or cortical nephrons would itself explain the reduction in concentrating ability, thereby making it unnecessary to invoke changes in medullary blood flow to account for this alteration in tubular function before a reduction in GFR. Rowe *et al.*. (1976), citing observations previously discussed, have suggested that a relative increase in medullary blood flow per nephron with age would result in enhanced removal of solute ("washout") from the medullary interstitium, and thereby decrease maximal urinary osmolality.

Elderly persons respond normally to graded doses of antidiuretic hormone (Pitressin®) insufficient to maximally concentrate the urine (Lindeman *et al.*, 1976). However, maximum urine osmolality following high dose pitressin infusion was significantly diminished in elderly subjects undergoing a water diuresis (Miller and Shock, 1953). This decrease appears most likely to be a result of diminished medullary tonicity rather than a defect in the ability of the kidney tubule to respond to pitressin. Goddard *et al.* (1984) showed in an in vitro renal cell suspension in mice a lack of cyclic AMP output in response to vasopressin which they speculated might play a role in the age-related decline in concentrating ability seen in experimental animals and elderly persons.

Maximum diluting ability, as measured by minimum urine osmolality, achieved with water loading, also decreases with age (Lindeman *et al.*, 1966). Another way to compare diluting ability in young versus old subjects would be compare free water clearance (C_{H_2O}) corrected for nephron mass as shown in Table 2. Although free water clearance decreases with age, C_{H_2O} factored by inulin clearance changes little with age, suggesting there is no basic defect in ability of the nephron tubule to produce a dilute urine.

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Urine acidification

Despite the decrease in renal function with age, the blood pH, pCO_2, and bicarbonate of aged persons without renal disease do not differ from the values observed in young subjects under basal conditions (Adler *et al.*, 1968; Shock and Yiengst, 1948). The decreases in blood pH and bicarbonate concentrations are prolonged, however, in elderly persons following ingestion of an acid load (Adler, *et al.*, 1968; Shock and Yiengst, 1948; Hilton *et al.*, 1955). The minimum urine pH achieved after an acid load is similar in young and old subjects, but total acid excretion (ammonia plus titratable acid minus bicarbonate) in ag-

TABLE 2. MEAN MAXIMUM DILUTING ABILITY (±S.D.) IN YOUNG, MIDDLE AGE AND ELDERLY MALE SUBJECTS AFTER INGESTION OF 20 CC H_2O/KG BODY WEIGHT (LINDEMAN, *et al.*, 1966)

	Young	*Middle Age*	*Old*
No. subjects	7	7	7
Mean age (years)	31	60	84
Mean GFR (cc/min)	149 ± 9	92 ± 8	65 ± 4
Urine Flow (cc/min)	19.8 ± 1.7	11.1 ± 1.5	8.5 ± 1.2
Urine osmolality (mOsm/L)	52 ± 3	74 ± 6	92 ± 11
Total solute excretion/100 cc GFR	690	840	1120
C_{H_2O} (cc/min)	16.2 ± 1.4	8.4 ± 1.3	5.9 ± 1.0
C_{H_2O}/100 cc GFR (cc/min)	10.9	9.1	9.1

ing persons decreases at a rate paralleling the decrease in glomerular filtration rate (Adler *et al.*, 1968).

Table 3 compares data obtained from young and old subjects after a standardized acid load. After baseline studies to define acid-base status of urine and blood, each patient ingested 0.1 g of ammonium chloride per kg body weight and serial blood and urine samples were obtained over the next 8 hours. The mean decrease in serum bicarbonate concentrations was nearly identifical in both groups (4.6–4.8 mEq/L). The minimum urine pH achieved in each age group was similar. A much larger percentage of the ingested acid load as measured by total acid excretion (ammonium plus titratable acid minus bicarbonate) was excreted by the young subjects compared to the older subjects. If total acid excretion is factored by glomerular filtration rate, similar values are obtained. The young subjects excreted a greater percentage of their total acid load as ammonium than did the older subjects, presumably because there was an increase in the urinary buffers responsible for titratable acid (phosphate, creatinine, etc.) per unit of glomerular filtration rate in the older subjects. Agarwal and Cabebe (1980) subsequently reported that elderly subjects showed a small pH gradient defect and ammonium excretion was reduced even after correction for glomerular filtration rate.

TABLE 3. MEAN ± S.D. INULIN CLEARANCE, MINIMUM URINE pH AND ACID EXCRETION IN RESPONSE TO A STANDARDIZED ACID LOAD (0.1 GM NH_4 Cl PER KG BODY WEIGHT) IN YOUNG VS. OLD NORMAL (ADLER *et al.*, 1968)

	Young	*Old*
No. subjects	10	10
Age range (years)	17–35	72–93
Inulin clearance (cc/min)	115 ± 16	55 ± 18
Minimum urine pH	4.96	4.85
Total acid excretion over a 8 hours (μEq/min)	100	39
Total acid excretion/100 cc/min GFR (μEq/min)	78	86
Percent of acid load excreted in 8 hours	35 ± 6	18 ± 5
Percent of total acid excreted as NH_4+	72 ± 7	59 ± 5

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Glomerular permeability

Essentially no information exists to indicate that glomerular permeability changes with age in man. Van Zonneveld (1959) found an increasing incidence of proteinuria in a population survey of persons over 65 years, nevertheless, by age 85, only a minority of patients had evidence of proteinuria. Glomerular permeability to free hemoglobin, determined by factoring free hemoglobin clearances by inulin clearances in healthy young and old subjects, did not change with age (Lowenstein *et al.*, 1961). Furthermore, there was no difference in glomerular permeability to a spectrum of different molecular weight dextrans between young and old subjects (Faulstick *et al.*, 1962). Finally, Arturson *et al.* (1971) reported that the filtration characteristics of intact glomeruli showed no evidence of changing glomerular pore size with increasing age.

AGE RELATED PROTEINURIA AND GLOMERULAR LESIONS IN RODENTS

In contrast, a number of reports have described age-related chronic glomerular lesions and increasing proteinuria in rats, hamsters, and mice (Sims and Berg, 1957, Everett,

1958; Guttman and Cohn, 1960; Berg, 1965; Perry, 1965; Beauchene *et al.*, 1970; Alt *et al.*, 1980, Bolton and Sturgill, 1980). These appear to be accompanied by age-related decreases in the transport of alpha-amino isobutyric acid and paraaminohippurate in kidney slices (Adams and Barrows, 1963; Beauchene *et al.*, 1965) and a decrease in concentrating ability (Burich, 1975).

Restriction in protein and total caloric intake delays the increase in proteinuria (Bras and Ross, 1964; Friend *et al.*, 1978) and the decreases in renal function (Tucker *et al.*, 1976) observed in aging rats and mice. Electron microscopic techniques have shown that there is a significant age-related increase in glomerular diameter and in the quantity of microvilli on the podocyte surface (Johnson and Barrows, 1980; Johnson and Cutler, 1980). The most recent studies by Bell *et al.* (1984) quantify the changes associated with spontaneous nephropathy in Lewis strain rats showing progressive increases in glomerular basement membrane thickness and mesangial area. Ultimately glomerular hyalinization and sclerosis occur. The tubulo-interstitial inflammation and fibrosis develops later and independent of the glomerular lesions. Protein restriction (Johnson and Barrows, 1980) and hypophysectomy (Johnson and Cutler, 1980) prevent the increases in abnormal morphology, thus diminishing the severity of the proteinuria. The development of age-associated proteinuria and renal disease has been studied in male Wistar rats fed varying caloric intakes and variable amounts of protein, carbohydrate and fat (Everitt *et al.*, 1982). Proteinuria developed more rapidly, kidneys were larger and the incidence of glomerular lesions and proteinaceous casts were greater in rats fed high calorie and high protein intakes.

Finally, O'Donnell *et al.* (1985) showed that age is a determinant of the glomerular morphologic and functional responses to chronic nephron loss. Four weeks after unilateral nephrectomy, young and adult rats had similar compensatory renal growth and single nephron function. However, after 5 months, the young animals showed significantly greater compensatory growth but single nephron function remained similar in both groups. The older animals had higher mean transcapillary hydraulic pressure differences. Although both age groups experienced accelerated expansion of mesangial matrix, significant glomerulosclerosis was observed only in the younger animals suggesting glomerular injury after nephrectomy may result from factors other than alterations in glomerular hemodynamics.

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Changes with age in the renal response to alterations in the internal and external environment

The changing responses of the kidney with age to various stimuli are more often dependent on different inputs to the kidney through extrarenal regulatory mechanisms than to internal changes in the kidney. The diurnal variations in urine sodium, potassium and chloride excretion and glomerular filtration rate appear to be blunted in older subjects when compared to younger subjects (Lobban, 1967). One potential explanation might be that the normal daytime increases in electrolyte excretion and glomerular filtration rate might be reduced by an exaggerated counterinfluence of posture in the older subjects, i.e. older persons might decrease electrolyte excretion and glomerular filtration rate more with standing than do young persons.

Actually when the response to tilt is compared in young and old subjects, there is little difference in the percent decrease in sodium excretion and glomerular filtration rate in

response to one hour of 45° tilt, i.e. there is no exaggerated response to tilting in aged subjects (Lee *et al.*, 1966).

Little information is available on the ability of the aging kidney to regulate sodium excretion in response to volume contraction and expansion. The aged individual can conserve sodium under conditions of acute dehydration (Sporn *et al.*, 1962), but the efficacy of this conservation has been compared critically in young versus old subjects only recently. Epstein and Hollenberg (1976) found that older subjects failed to conserve sodium as rapidly and efficiently as did younger subjects. Weidman *et al.* (1975) and Crane and Harris (1976) have shown that elderly persons have lower plasma renin activities (PRA) and urinary aldosterone excretions both on an unrestricted salt diet and after sodium restriction (3–6 days) than do young subjects. Salvetti *et al.* (1980) found this inverse relationship in the supine position between plasma renin activity and age was lost when a controlled normal sodium diet was ingested. It became apparent only in the upright position and when sodium intake was reduced. They believed that decreased sympathetic nervous system activity at least partially explained the age influence on the PRA-sodium relationship. Karlberg and Tolagen (1977) showed that, although basal plasma renin activities were lower in old than in young subjects, the increase with ambulation (upright PRA) and after 80 mg of frurosemide (stimulated PRA) were not different in the young and old. Serum cortisol and renin substrate concentrations, in contrast, were not different in young and old subjects. Urinary sodium retention on a low salt intake was less impressive in old than in young subjects. These data support earlier studies reported on changes in plasma renin concentrations (Hayduck *et al.*, 1973) and urinary aldosterone excretions (Flood *et al.*, 1967) observed with age. The cause for the decrease in PRA and aldosterone excretions remains speculative. No correlation was evident when these measurements were compared with blood pressure levels so that altered systemic pressure could not be implicated. Furthermore, no relationship existed between postural renin response and accompanying blood pressure changes in young versus old subjects. Nephrosclerosis or other morphologic or functional alterations or a decrease in sympathetic activity may in some way lead to a reduction in renin release and subsequent aldosterone excretion. Regardless of the mechanism, the decreased PRA and aldosterone excretions probably account for the decreased ability of elderly subjects to conserve sodium when challenged with a low salt diet. Elderly male subjects are more likely to develop an exaggerated natriuresis after administration of a water or saline load than are younger male subjects (Lindeman *et al.*, 1970). Schalekamp *et al.* (1971) found that older hypertensive patients in whom renal vascular resistance and filtration fraction were increased and plasma renin concentrations were suppressed, consistently developed a more marked natriuresis following an intravenous saline load than did younger hypertensive patients. The ability of the aged person to eliminate large amounts of sodium when stressed with excessive salt intake remains poorly defined.

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The kidneys are responsible for maintenance of a constant internal environment of fluid volume and electrolyte concentrations. Imbalances result not from intrinsic renal changes with age but rather from extrarenal pathology affecting renal regulatory mechanisms. Cohn and Shock (1949) showed no change with age in total blood volumes or plasma volumes in a large number of male subjects, age 20 to 90 years. Serum sodium, potassium, chloride, calcium and magnesium concentrations do not vary significantly with age (Elkinton and Danowski, 1955; Shock, 1961; Korenchesky, 1961) nor does acid-

base balance as discussed earlier. The significance of small, but statistically significant decreases in serum zinc concentrations (Lindeman *et al.*, 1971) and increases in serum copper concentrations (Yunice *et al.*, 1974) in males with age remain to be defined, but it is unlikely that the changes are related to any intrinsic renal alteration with age.

Armbrecht *et al.* (1980) recently have demonstrated a marked decrease (10-fold) in the ability to convert 25-hydroxy-vitamin D_3 to 1, 25 dihydroxy D_3 in intact young (1.5 month) compared to adult (12 month) rats. The decreased ability to 1-hydroxylate 25-hydroxy D_3 also was observed in isolated slices of rat kidney.

COMPENSATORY RENAL HYPERTROPHY

Unilateral nephrectomy causes compensatory hypertrophy in a normal, remaining kidney. Renal size and function increase rapidly the first three days to one week, more slowly the subsequent three weeks, and then may show continued slight increases for many months (Boner *et al.*, 1973). MacKay, MacKay and Addis (1926, 1932) were the first to demonstrate that the magnitude of compensatory hypertrophy decreases with age at the time of nephrectomy. In rats aged 1, 2, 9, 12 and 18 months the increases in residual kidney weight were 44, 35, 33, 23, and 23%, values almost identical with those found by many subsequent investigators (Tauchi *et al.*, 1958; Odgen, 1967; Verzar, 1954; Barrows *et al.*, 1962; Reiter *et al.*, 1964; Phillips and Leong, 1967; Reiter *et al.*, 1964; Reiter *et al.*, 1964; Phillips and Leong, 1967; Dicker and Shirley, 1973; Galla *et al.*, 1974). Compensatory hypertrophy is directly related to protein intake, but the age differences persist when this variable is controlled (McKay *et al.*, 1932; Konishi, 1962; Kennedy, 1957).

The number of glomeruli does not increase after early life in the rat or after birth in man, and the compensatory enlargement is not due to an increase in the number of nephrons (Arataki, 1926; Moore and Hellman, 1930; Moore, 1929; Kaufman *et al.*, 1975), although one recent dissenting report (Bonavalet, 1972) noted an increase in the number of glomeruli in rats nephrectomized under 50 days of age. The glomeruli increase in volume and the tubules enlarge, chiefly in the proximal convoluted segment (Konishi, 1962; Kennedy, 1967; Mandache and Repciuc, 1975; McCreight and Sulkin, 1959). In young animals cellular hyperplasia predominates; in older individuals the chief response is cellular hypertrophy (Tauchi *et al.*, 1958; Reiter *et al.*, 1964; Phillips and Leong, 1967; Dicker and Shirley, 1973; Kennedy, 1957; Zumoff and Pachter, 1968). This is supported by evidence from several sources, cell counts (Tauchi *et al.*, 1958; Zumoff and Pachter, 1964), RNA/DNA ratios (Dicker and Shirley, 1973; Kennedy, 1957; Johnson and Vera Roman, 1966), counts of mitoses (Reiter *et al.* 1964; Phillips and Leong, 1967; McCreight and Sulkin, 1959), measures of double stranded DNA replication (Levitsky, 1980), and autoradiography (Verzar, 1954). These studies uniformly demonstrate that although the timing and functional responses in young and old individuals are similar, the proliferative activity is greatly reduced in animals beyond six months of age. The only discordant report is that of Konishi (1962), who found higher mitotic indices in old animals. A seeming discrepancy in the report of Barrows *et al.* (1962), who reported no change in the RNA/DNA ratios, is explained by their use of 12 and 21 month old rats, since both groups are beyond the age of significant proliferation shown by other studies. Probably relevant are the kidney epithelial cell cultures reported by Soukupova *et al.* (1965), who found active growth of cells from newborn rats, but very poor and poorly sustained

growth of cells from animals beyond 8 months. They conclude that aging is a property of the cells themselves, aggravated by further changes in the whole organism. Rickets *et al.* (1985) showed that the rate of total protein synthesis by suspensions of kidney cells declined 60% in rats between 4 and 31 months of age. They felt this decline in protein synthesis with age, which correlated inversely with the level of proteinuria, preceded the development of the renal disease observed in this species. Furthermore, dietary protein restriction decreased the rate of protein synthesis and decreased the level of proteinuria. Moore and Heilman (1930) noted that compensatory hypertrophy had no effect upon the decrement in nephron number with age.

Renal compensatory hypertrophy has been of recent clinical interest due to the success of kidney transplantation. Ogden (1967) found that in 28 donors the remaining kidney showed an inverse relationship between age at the time of nephrectomy and function three years later. The correlation between GFR and donor age was $r = -0.53$, $p < 0.01$, and between ERPF and donor age was $r = -0.43$, $p < 0.05$. The inverse relationship of age to the ratio of postoperative to preoperative ERPF was highly significant ($r = -0.65$, $p < 0.01$) for the 17 donors who had both pre- and post-transplantation evaluations. In a more elaborate study by Boner *et al.* (1973), a total of 259 examinations were performed on 49 donors, 22 females and 27 males, 21 to 63 years of age. The greatest functional response occurred in one to three weeks, and was comparable at all ages. There was then a continuing, slower increase which continued at least four years, yielding a final single kidney GFR increment of 48% at 20 years and 30% at 60 years, with a linear distribution at intermediate ages. Slack and Wilson (1976) report no significant age related difference in compensatory function, although their data show some differences at the extremes of the ages studied.

In general, compensatory hypertrophy has been measured by kidney weight gain in rats and single kidney increments in function in man. The results correlate well. Two more recent studies (Galla *et al.*, 1974; Kaufman, 1975) demonstrate in rats that increases in structure and function are parallel.

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PATHOPHYSIOLOGY OF THE DECREASE IN RENAL FUNCTION WITH AGE

How does kidney function decrease with age in the individual subject? Cross-sectional studies from "normal" aging populations demonstrate a consistent decrease in renal function with age which tends to accelerate in the oldest age groups. Is this decrease due to a progressive involutional change with loss of nephron units and a decline in cellular function through the life of the individual, or does renal function remain stable until intermittent pathologic processes produce multiple, intermittent acute or chronic decreases in renal function? Subclinical or silent renal injuries, e.g. undetected glomerulonephritis due to immunological injury following an infection, pyelonephritis due to bacterial or viral infection, acute tubular injury or interstitial nephritis due to drug reaction, poison or acute illness, vascular occlusion with resultant ischemic injury and urinary tract obstruction, would be examples of such "events". What role does the deceasing ability to repair and replace injured and destroyed cells in the nephron play in the accelerating decrease in renal function with age? These are questions which can only be partially answered with available data and will present a challenge for future researchers studying the aging process in the kidney.

EVIDENCE THAT RENAL CHANGES WITH AGE DUE TO PROGRESSIVE INVOLUTION

At one time, cells placed in culture media were believed to multiply indefinitely if proper cultural conditions were provided. Cells capable of unlimited in vitro activity are invariably abnormal, often resembling cancer cells. Studies by Hayflick (1973, 1975, 1976) have shown that normal cells, such as diploid human fibroblasts, have a finite life span and that the population-doubling potential of these cells is inversely related to the age of the donor. Furthermore, there is no evidence that normal cells can be maintained in a state of active proliferation or even in a functional state in cell culture for a period of time in excess of the specific age of the species from which the cells were obtained.

Similar observations have been made of renal cells cultured in vitro (Soukupova *et al.*, 1965). The previously described uniform failure of older kidneys to manifest compensatory hypertrophy by cellular hyperplasia further supports the concept of cellular change with age.

As cultured normal cells approach the end of their in vitro lifetime, a number of biochemical decrements occur which herald the approaching loss of division capacity (Hayflick, 1973; Hayflick, 1975; Hayflick, 1976). The animal studies reported earlier showing fewer energy producing mitochrondria, lower enzyme concentrations, lower concentrations of sodium-potassium activated ATPase activity, decreased sodium extrusion and oxygen consumption, and diminished tubular transport in old compared to young kidneys would be consistent with such a cellular aging phenomenon. Whether cellular changes in aging animals and ultimately man can be related to the physiological manifestations of aging in the kidney (decreased glomerular filtration rate, renal blood flow, tubular maximums, and concentrating, diluting and acidifying abilities) remains to be documented.

The observation of glomerular loss in the absence of overt lesions in the large and small vessels in many studies indicates that nephron loss occurs normally without an overt vascular cause. The sequence described by Ljungvist (1963, 1964) and Takazakura *et al.* (1972) appears to originate at the glomerular level, possibly associated with a capillary lesion, and does not show evidence of the ischemic collapse described as typical of the larger vessel disease described by McManus and Lupton (1960).

It is difficult to attribute the change to specific disease entities in the absence of any characteristic observable pathologic abnormality. The age-related loss of tissue and function in many organ systems challenges the concept of system-specific disease entities acting in apparent concert. Similar vascular changes in the small vessels with reduced perfusion have for example been described in the brain (Fang, 1976) and in the testis (Sasano and Ichigo, 1969; Suoranta, 1971). These changes could, of course, be the result, as well as the cause, of a reduced cell mass requiring perfusion. Oliver's (1939) early observation that the metanephric kidney followed, in sequence, the embryologic senescence of the pronephros and the mesonephros, indicates that involution need not be the result of unnatural or extrinsic factors.

EVIDENCE THAT RENAL CHANGES WITH AGE ARE DUE TO SUPERIMPOSED PATHOLOGY

Freidman *et al.* (1972) utilized scintillation scanning techniques to localize defects in kidney function in elderly persons with no past history of renal disease. They found abnormal scans in 25 of 35 elderly patients (71%) with a mean age of 75 years (range 60–93

years) and mean creatinine clearance of 53 ml per minute. Sixteen (46%) showed focal areas of diminished uptake which were felt to represent ischemic lesions. Significant pyuria was present in 37% of the patients; however, intravenous pyelograms were interpreted as normal in all cases. No significant proteinuria was detected in any patient. These findings are consistent with the hypothesis that focal lesions due to vascular occlusions, interstitial infection (pyelonephritis) or both contribute to the decrease in renal function observed in aging persons.

Tauchi *et al.* (1971) compared postmortem renal pathology in elderly Caucasians and Japanese. Arteriosclerotic lesions appeared earlier and were more severe in the small arteries of the kidney in the Japanese compared to Caucasians consistent with the increased severity of the arteriosclerotic lesions found in other parts of the body. Kidney weight, size of glomeruli, number of cells per glomerular tuft, and number of tubular epithelial cells in a given area all decreased significantly with age and the decline was significantly more rapid in the Japanese. The size of the individual epithelial cell nuclei, however, increased significantly with age, and the increase was more apparent in the Caucasians. The authors concluded that sclerotic and fibrotic changes in the renal vasculature paralleling a generalized arteriosclerotic process were primarily responsible for the senile changes occurring in the kidney.

Asymptomatic bacteriuria also may be an important contributor to the decrease in renal function with age observed in cross-sectional studies. Dontas *et al.* (1968) found 24 of 90 (27%) clinically healthy residents of the Athens Home for the Aged had persistent bacteriuria. The mean inulin clearance was significantly lower (70 vs. 81 cc/min) in the bacteriuric group when compared to other residents. Concentrating ability also was decreased more in the bacteriuric residents. Wolfson *et al.* (1965) found asymptomatic bacteriuria in 15% of 521 geriatric male patients (median age 63 years). The age-related incidence of bacteriuria was relatively constant at 9% up to age 60 years; it then increased rapidly reaching 42% in individuals over age 80 years. Although the incidence of prostatic hypertrophy, urinary calculi, previous infection, instrumentation, and surgery increased with age, there were some bacteriuric individuals in whom these predisposing conditions were not found.

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The importance of immune injury as a cause of renal morphologic changes with age also remains unclear. Both rats and mice (Couser and Stilmant, 1976; Shimizu *et al.*, 1977) show increasing accumulations of fluorescent staining antibodies and complement in the glomeruli with age but this does not confirm that there exists an antigen-antibody-complement-mediated form of immune injury. Couser and Stilmant (1976) described a marked increase with age in mesangial deposition of IgM-containing macromolecular materials with the onset on proteinuria beginning about 12 months of age. Aging rats subsequently developed focal and segmental glomerular sclerosis and these sclerotic lesions stained diffusely with several immunoglobulins. Since they found no autoantibody activity in the circulation or kidneys, they felt that autoantibody production and immune complex deposition do not appear to mediate the glomerular changes associated with aging in the rat kidney. Rather, deposition of macromolecular material in the glomerular mesangium as a consequence of the increase in glomerular permeability may result in a functional alteration of normal mesangial phagocytic or clearing mechanisms that contributes in the development of the glomerular sclerosis of aging in the rat.

Studies reported from the Baltimore Longitudinal Study of Aging on a cohort of male volunteers followed between 1958 and 1981 provide more evidence that the decrease in renal function observed with age may be the result of intervening pathology rather than a

relentless involutional process (Lindeman *et al.*, 1984, 1985). One third of the 446 subjects with 5 or more studies showed no decrease in renal function (positive slope of creatinine clearance vs. time). Furthermore, there was a small group of subjects, even some elderly persons, who showed statistically significant increases ($p < 0.05$) in creatinine clearances with age.

As discussed earlier, when one kidney is removed, the opposite kidney develops compensatory hypertrophy. A humoral mechanism is known to exist to stimulate growth through hyperplasia and hypertrophy. This process originally was felt to be activated only when there was a functional loss of kidney as a compensatory mechanism. Subsequently, it was recognized that both kidney size and glomerular filtration rate are increased in early onset diabetes (Kahn *et al.*, 1974; Mogensen, 1976) so that the process appears to become operable under conditions other than loss of renal function. The glomerular filtration rate returns to normal when the abnormal glucose metabolism is corrected. When a state of glomerular hyperfiltration and/or hyperperfusion is created by abnormal glucose metabolism, by increasing protein intake or blood pressure (hypertension), or by decreasing surviving nephrons, an accelerated nephrosclerosis develops that thereafter lowers filtration rate (Brenner *et al.*, 1982; Klahr, *et al.*, 1983.). This process may not only explain the accelerated loss of renal function observed in diabetics and hypertensives, but may apply to the accelerated decline in renal function observed in elderly subjects (Brenner *et al.*, 1982).

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URINARY DILUTING CAPACITY IN ELDERLY DIABETIC SUBJECTS

FAITH B. DAVIS, ALLENE VAN SON, PAUL J. DAVIS, and LEILA EDWARDS

Endocrinology Division, Department of Medicine, State University of New York at Buffalo School of Medicine, the Erie County Medical Center, the Erie County Laboratory, Buffalo, NY, and the Veterans Administration Medical Center, Buffalo, NY

Abstract—Urinary concentrating ability declines with normal human aging but diluting capacity has been less well studied as a function of age. We studied free water clearance (C_{H_2O}) in a group of Type II diabetic patients, aged 47 to 70 years. Conventional water load testing in sulfonylurea-treated diabetic patients revealed preservation of diluting capacity (ability to achieve U_{osm} <100 mOsm/kg) in subjects >60 years of age. Tolazamide permitted expression of normal C_{H_2O}; chlorpropamide administration predictably reduced C_{H_2O} relative to tolazamide by 58% in subjects >60 years. Free water clearance (C_{H_2O}) and glomerular filtration rate (GFR) were comparable in elderly subjects (>60 years) and those ≤60 years. Normal C_{H_2O} in these diabetic patients was explained by persistently normal GFR in the study group. Osmolar clearance (C_{osm}) was insignificantly lower in diabetics >60 years. Thus, a fall in C_{H_2O} in elderly diabetic patients is not a factor contributing to clinical hypoosmolar states encountered in this population.

Key Words: diabetes, urinary diluting capacity, aging, water clearance

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URINARY CONCENTRATING ability declines as a function of age in man (Lindeman *et al.*, 1966; Rowe *et al.*, 1976b). This change is accompanied by heightened osmoreceptor sensitivity in elderly subjects and increased arginine vasopressin (AVP) secretion, compared to younger individuals, in response to osmotic stimuli (Helderman *et al.*, 1978). Urinary diluting ability, the ability to increase free water clearance (C_{H_2O}) appropriately in response to hypoosmolar stimulus, is less well-studied as a function of normal aging (Lindeman *et al.*, 1966; Lye, 1984). An age-dependent decrease in glomerular filtration rate (GFR) (Rowe *et al.*, 1976a), however, should increase the solute load per nephron and—unless osmolar clearance falls on an age-related basis in proportion to GFR—limit diluting capacity. We have been interested in water metabolism in diabetes mellitus (Davis, F. and Davis, P., 1981) and in factors which contribute to clinical hypoosmolar states (Davis, F. *et al.*, 1982). In the present study we have examined GFR and urinary diluting capacity in sulfonylurea-treated Type II diabetic subjects to determine if diluting capacity was impaired in this group of elderly patients. Impaired C_{H_2O} in this group is a

Correspondence to: Dr. Paul J. Davis, Erie County Medical Center, Buffalo, NY 14215.
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risk factor for the development of serum hypoosmolarity with water-loading or with the use of thiazides (Fichman *et al.*, 1971), chlorpropamide (Garcia *et al.*, 1971; Weissman *et al.*, 1971) or other drugs (Miller and Moses, 1976) which promote hyponatremia.

SUBJECTS AND METHODS

Patients

Eighteen diabetic patients, ranging in age from 47 to 70 years (Table 1), provided informed consent to this Institutional Review Board-approved protocol. The subjects were classified as Type II diabetics on the basis of histories free of ketoacidosis and responsiveness of hyperglycemia to oral sulfonylurea therapy. The patients were healthy at the time of study except for variable degrees of hyperglycemia and glycosuria (Table 1). Serum creatinine concentrations were ≤ 1.5 mg/dl in all subjects; individual subjects' endogenous creatinine clearances are shown in Table 1. There was no significant difference between subjects ≤ 60 years (Group I) and those >60 years (Group II) with respect to serum creatinine, endogenous creatinine clearance or 24 h urine glucose, although the latter was higher in Group I. The fasting serum glucose was significantly higher in Group I subjects than in Group II subjects ($P < 0.05$).

TABLE 1. CLINICAL DATA

Subject	*Age (yrs)*	*Sex*	*Fasting Serum Glucose (mg/dl)*	*Urinary Glucose (g/24 h)*	*Serum Creatinine (mg/dl)*	*Creatinine Clearance (ml/min)*
I. 1	50	F	304	23.62	1.1	73.0
2	57	F	178	0.76	1.2	60.0
5	60	M	270	66.73	1.2	105.8
9	59	M	103	0.26	1.0	111.2
14	47	F	288	24.28	0.8	170.3
15	56	M	273	172.35	1.1	98.2
17	60	M	111	0.48	1.1	129.4
18	58	F	204	2.60	0.8	92.2
$n = 8$	55.9 ± 1.7		216 ± 21	36.38 ± 15.16	1.0 ± 0.1	105.0 ± 8.9
II. 3	61	M	94	0.48	0.9	80.0
4	62	M	162	2.80	1.1	83.2
6	67	F	234	17.69	1.2	64.2
7	66	M	151	4.18	1.1	99.7
8	67	F	273	43.08	0.8	83.9
10	61	M	159	0.53	1.1	93.2
11	70	M	191	3.89	0.9	110.6
12	68	M	159	15.60	1.5	79.9
13	65	F	114	0.24	0.8	96.7
16	67	M	104	0.34	0.7	131.9
$n = 10$	65.4 ± 1.0		164 ± 13*	8.88 ± 3.61	1.0 ± 0.1	92.3 ± 4.4

*$P < 0.05$ comparing serum glucose values in two age groups by t test.

Study design

Each subject underwent two water load tests; these were carried out at the conclusion of each of two 4-week periods in which the subjects received one oral sulfonylurea (chlorpropamide or tolazamide) in the first period, and the alternate sulfonylurea in the second period. Twenty-four hour urinary glucose and creatinine excretions were determined on the day prior to water load testing; serum samples were obtained at the beginning of each water load test.

Free water clearance (C_{H_2O}) and osmolar clearance (C_{osm}) measurements

C_{H_2O} and C_{osm} were determined after a conventional oral water load (20 ml/kg body weight) as previously described (Davis, F. *et al.*, 1982). The C_{H_2O} was corrected for GFR by the formula: corr. $C_{H_2O} = C_{H_2O} \times 100/C_{creatinine}$. The minimum urine osmolality (U_{osm}) achieved during the test was also recorded. Patients were tested in the morning after an overnight fast. They ingested 500 ml of water upon arrival in the laboratory and 30 min later received their water load. Serum and urine osmolalities were measured by freezing point depression. In all subjects basal serum osmolality was greater than 280 mOsm/kg and serum sodium concentration was normal (>135 mEq/L).

RESULTS

Maximum urinary diluting capacity

Maximum diluting ability in response to water-loading was defined as minimum U_{osm} achieved (Table 2). A normal response to formal water-loading is generation of U_{osm} of <100 mOsm/kg (Moses *et al.*, 1985). Age-specific analysis of data showed that patients over age 60 years performed as well as those under age 60 years (Table 2) and both groups achieved minimum U_{osm} values of <90 mOsm/kg. For 13 of 18 subjects, the minimum U_{osm} was achieved during management of glycemia with tolazamide, an oral sulfonylurea

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TABLE 2. RESULTS OF WATER LOADING TESTS.*

		Subjects ≤ 60 years		*Subjects > 60 years*	
		Chlorpropamide	*Tolazamide*	*Chlorpropamide*	*Tolazamide*
Minimum U_{osm}	mOsm/kg	159.9 ±49.8	88.0 ±8.5	144.2 ±16.4	87.8** ±8.1
C_{H_2O}	ml/min	2.59 ±0.88	3.04 ±0.58	± 1.54 ±0.47	3.45** ±0.59
C_{H_2O} (corrected for GFR)	ml/min	2.57 ±0.92	2.94 ±0.65	1.59 ±0.43	3.77** ±0.64
C_{osm}	ml/min	2.81 ±0.26	2.84 ±0.39	2.54 ±0.22	2.19*** ±0.19

*Results shown are means ± SE of studies on a total of 18 subjects; 8 were ≤ 60 years and 10 were > 60 years old. Each subject completed two water load tests, each test after four weeks of treatment with each of two sulfonylureas.

**$p < 0.02$ calculated by paired t test comparing tolazamide with chlorpropamide.

***$p < 0.05$ by paired t test.

which does not impair C_{H_2O} (Moses *et al.*, 1973). In Group II subjects >60 years, but not in subjects ≤60, there was a significant difference in minimum U_{osm} in the two treatment periods, with mean minimum U_{osm} of 87.8 and 144.2 mOsm/kg achieved during tolazamide and chlorpropamide therapy, respectively. Control of glycemia (serum glucose at time of study <200 mg/dl, below the conventional tubular maximum for reabsorption of glucose) and absence of thiazide administration did not significantly improve minimum U_{osm} achieved (data not shown).

Free water clearance

C_{H_2O} results, uncorrected and corrected for GFR and shown in Table 2, indicated that maximum performance was independent of subject age. The higher C_{H_2O} in the two tests was achieved during tolazamide therapy in 13 of 18 patients. Measurement of corrected C_{H_2O} in subjects >60 years, treated with chlorpropamide as compared with tolazamide, showed a substantive difference in performance, with C_{H_2O} 58% reduced in chlorpropamide-treated subjects (Table 2). This difference was not evident in Group I subjects, in whom a greater osmotic diuresis may have blunted the effects of both sulfonylureas on C_{H_2O} (Gennari and Kassirer, 1974). Thiazide administration had the effect of enhancing the antidiuretic effect of chlorpropamide (Davis, F. *et al.*, 1982). Hyponatremia or hypoosmolarity did not occur in any subject as a consequence of water-loading.

Osmolar clearance

C_{osm} was 16% lower in patients >60 years than in patients ≤60 years; this change, although not statistically significant, was probably related to increased glycosuria of subjects in Group I as compared with Group II.

DISCUSSION

Results of studies of urinary concentrating ability in subjects of varying age have shown an age-dependent decline in this function (Lindeman *et al.*, 1966; Rowe *et al.*, 1976b). In a previous analysis of urinary diluting capacity, Lindeman (1966) reported in a small group of apparently healthy older subjects a decreased ability to maximally dilute urine after water loading. The current studies, conducted in a series of Type II diabetics over age 45 years, has shown in contrast that urinary diluting capacity may be preserved in older subjects. The patients studied here were diabetic and had normal creatinine clearances. In the absence of thiazide diuretic therapy but with fasting serum glucose levels below 200 mg/dl, these subjects, when tolazamide-treated, achieved a mean U_{osm} of <90 mOsm/kg upon conventional water-loading. While this performance is normal, it is also important to point out that it is not maximal performance, since serum hypoosmolarity was not observed during these water loading studies (Davis, F. *et al.*, 1982); it is likely, therefore, that further diluting capacity ("reserve") existed in the study group. In the chlorpropamide administration phase of the study, C_{H_2O} was modestly reduced relative to tolazamide treatment; chlorpropamide is well known to have this effect in younger subjects (Moses *et al.* 1973). Dose-response studies were not carried out in the present protocol to determine the threshold of the chlorpropamide effect on C_{H_2O}. It is clear, however, that low doses of the drug in Type II diabetic patients can induce hyponatremia (Weissman *et al.*, 1971).

In order for C_{H_2O} to have been preserved in the elderly patients studied, one or more of the following sequences must have occurred: 1) GFR was preserved; 2) GFR fell but C_{osm}

fell to a degree sufficient to offset the decline in GFR; 3) there was heightened ability in elderly subjects to turn off secretion of AVP. Helderman *et al.* (1978) have reported to the contrary, that healthy elderly subjects show incomplete suppression of AVP in response to ethanol infusion. No data are available in the current study relevant to AVP suppressibility. Persistence of normal GFR in this cohort underlies the normal response to water-loading we observed. The possibility exists that "preservation" of GFR in this group of elderly patients represents relative hyperperfusion, since an early index of diabetic nephropathy is increased GFR (Mogensen, 1976). This possibility has not been explored in any studies of elderly Type II diabetics, perhaps due to an assumption that nephrosclerosis in elderly diabetics, among other factors, precluded a hyperperfusion state. Table 1 shows that a downward trend in GFR in our older patient group was not significant. Osmolar clearance also decreased insignificantly; it is thus unlikely that reduced C_{osm} in our older subjects contributed materially to the achievement of appropriately dilute urine.

The difference in C_{H_2O} in the elderly reported by Lindeman and by us is, in fact, small. Lindeman did not study diabetic subjects and the presence of diabetes mellitus in this cohort and attendant risk of nephropathy caused us to expect an exaggerated decrease in C_{H_2O} in our subjects. Patient selection factors, aside from diabetes mellitus, may have favored decreased C_{H_2O} performance in Lindeman's study and normal performance in the present group. It should also be noted, however, that a more rigorous water load protocol was used by Lindeman which may have unmasked subtle age-dependent changes in maximum urinary diluting capacity.

While these observations require confirmation in a larger group of subjects, we would propose that a decline in C_{H_2O} is not a necessary concomitant of aging and that clinical hypoosmolar states in elderly diabetic patients do not depend upon age-dependent change in urinary diluting capacity.

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SPLANCHNIC FACTORS ENHANCE THE NOREPINEPHRINE RESPONSE TO ORAL GLUCOSE IN AGED MAN

RICHARD P. TONINO, KENNETH L. MINAKER, JAMES B. YOUNG, LEWIS LANDSBERG, and JOHN W. ROWE

Charles A. Dana Research Institute and the Harvard Thorndike Laboratory of the Beth Israel Hospital; Joint Department of Medicine, Beth Israel and Brigham and Women's Hospitals; The Division on Aging, Harvard Medical School; and the Geriatric Research Education Clinical Center, West Roxbury/Brockton Veterans Administration Medical Center, Boston, Massachusetts

Abstract—Oral glucose has been shown to increase sympathetic nervous system (SNS) activity more in old than in young subjects. In contrast intravenous glucose during euglycemic hyperinsulinemia increases SNS activity in young but not in old subjects. To evaluate the role of splanchnic factors in this discrepancy, we employed a modification of the glucose clamp technique in 6 young (24–39 years) and 8 old (65–83 years) normal males. Each subject underwent two studies in which insulin was infused at 120mU/m² • min for 3 h and either oral glucose (50 gms) or water was given 60 min after initiating insulin. Euglycemia was maintained in all studies. When compared to control drink, oral glucose elevated norepinephrine in old ($p < 0.01$), but not in young subjects. The difference between old and young was significant ($p < 0.02$). When compared to control drink, oral glucose increased pulse rate and double product in the young, and pulse rate in the old. These results indicate that oral glucose activates the SNS in the elderly via splanchnic mechanisms independent of changes in circulating levels of glucose or insulin.

Key Words: catecholamines, cardiovascular response, glucose clamp, carbohydrate ingestion

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INTRODUCTION

RECENT STUDIES have explored the link between carbohydrate intake and sympathetic nervous system (SNS) activity in man. The plasma norepinephrine (NE) response to oral glucose ingestion is greater and more prolonged in elderly as compared with young men (Young *et al.*, 1980). Studies employing the euglycemic insulin clamp technique which permit examination of the effects of hyperinsulinemia independent of hyperglycemia have demonstrated enhanced sympathetic nervous system activity during hyperinsulinemia in young adults (Rowe *et al.*, 1981). In the elderly, however, this stimulatory effect of hyperinsulinemia is absent (Minaker *et al.*, 1982). The present study was designed to examine the mechanism underlying the apparent discrepancy with age between the response

Correspondence to: John W. Rowe, M.D., Gerontology Division, Beth Israel Hospital, 330 Brookline Avenue, Boston, MA 02215.
(*Received* 14 *May* 1986; *Accepted* 27 *August* 1986)

to oral glucose and intravenous insulin with euglycemia. Our results indicate that in the elderly splanchnic factors raise plasma NE in response to oral glucose compared with a noncaloric control drink. This effect was independent of changes in serum insulin or blood glucose, and was significantly different from the young in whom oral glucose had no effect on plasma NE compared with control drink.

METHODS

Subjects

Six young (24–39) and 8 old (65–83) healthy male volunteers participated in these studies. Relative weight was 1.09 ± 0.04 (mean ± SE) (range 0.98–1.25) in the young and 1.14 ± 0.05 (range 0.92–1.27) in the old. Relative weight was calculated as the ratio of actual to desirable weight using the middle of the weight range for subjects of medium frame from the 1959 Metropolitan Life Insurance Company table for desirable weight. The elderly subjects were active, community dwelling middle class Caucasians who participate in the Normative Aging Study of the Veterans Administration (Bell *et al.*, 1972). The young subjects were community dwelling, nonmedical personnel. On screening, all subjects were free of intercurrent and chronic diseases, and were taking no medications. All had normal glucose tolerance and no family history of diabetes mellitus (National Diabetes Data Group, 1979). For 3 days before each study, subjects supplemented their usual diets to insure a daily carbohydrate intake of greater than 150 gm. Subjects were hospitalized on the evening before a test and studied the following morning in the fasting state. The protocols were approved by the Committee on Clinical Investigations, New Procedures and New Forms of Therapy at the Beth Israel Hospital, and informed consent was obtained from all subjects before study.

Glucose clamp

A previously described variant of the euglycemic hyperinsulinemic clamp technique was employed to allow 50 g glucose to be injested without altering the systemic blood glucose level (Anderson *et al.*, 1978) (Fig. 1). The general procedure for the clamp technique was as previously described (Defronzo *et al.*, 1979). Crystalline pork insulin was infused into an antecubital vein at 120 mU/m² • min for 140 min after an initial 10 min priming infusion. Glucose in water (20%) was started in the same vein at 4 min and adjusted every 5 min to maintain basal euglycemia for 180 min. Between 60 and 65 min, a glucose drink (50 g/350 ml water with lemon flavoring) or a noncaloric water drink (350 ml) was injested. Both solutions were 30°C when ingested. Subjects were studied in the semirecumbent position and no change in position occurred during or after the drink. Each subject underwent both glucose and control drink studies separated by at least 1 week. These paired studies were, therefore, controlled for oral fluid volume, euglycemia and insulin infusion rate. When glucose was the ingested drink, euglycemia was maintained by reducing the intravenous infusion of glucose by empirically determined formulas to compenstate for the post hepatic delivery of the orally administerd glucose (Anderson *et al.*, 1978).

Subjects were awake throughout all studies. Arterialized venous blood samples were drawn from a retrogradely catheterized dorsal vein of a hand which rested in a warming chamber (68°C) throughout the study (McGuire *et al.*, 1976). Forty-five minutes after insertion of IV catheters and prior to initiation of the insulin infusion, two basal samples

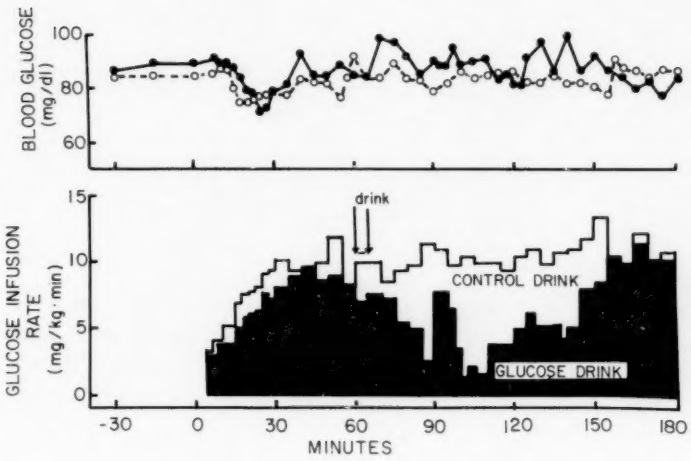

FIG. 1. Euglycemic hyperinsulinemic (120 mU/M² • min) clamp studies in a 72-year old subject illustrates the IV-oral technique. Blood glucose and IV glucose infusion rate over time for control drink (○) and 50 gm glucose drink (●) studies.

were obtained for glucose, insulin and NE. Blood glucose was then determined at 5 min intervals, and insulin and NE at uniform intervals both before and after the oral drinks. In addition, pulse rate and blood pressure were measured by arm cuff technique at regular intervals throughout all studies. Pulse pressure was calculated as systolic minus diastolic BP, and mean arterial BP as diastolic BP plus one-third of the pulse pressure. Double product was calculated by multiplying pulse rate and systolic BP and used as a noninvasive correlate of myocardial oxygen consumption (Kitamura *et al.*, 1972).

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Analytical methods

Blood glucose and serum insulin were handled and analyzed as previously described, with insulin from different experiments on the same subject determined in the same assay (Rowe *et al.*, 1981). Plasma NE was measured by liquid chromatography with electrochemical detection as previously described (Davis *et al.*, 1983). The lower limit of sensitivity in our laboratory is 10–15 pg/ml. The intra-assay coefficient of variation at basal levels is 6–8%. Total body glucose disposal was calculated as the amount of glucose infused during the steady state period 60–180 min minus glucose space corrections and is a measure of peripheral tissue uptake of glucose. Hepatic glucose production has been shown to be equally suppressed in young and old men under hyperinsulinemic conditions employed in this study (Fink *et al.*, 1983).

Data are presented as mean ± SE. Statistical analysis employed repeated measures analysis of variance (ANOVA) for comparisons between glucose and control drinks within an age group, and between old and young for the effect of the glucose drink (Jennrich, 1981). To account for person to person variability, pretreatment levels of each variable were used as a covariate in the repeated measures ANOVA in comparing responses to the glucose and control drinks. All analyses were carried out on absolute values. For NE the mean of the two NE levels preceding a drink (+50 and +60 min) was

employed as the covariate. For pulse rate, pulse pressure, mean arterial BP and double product, the mean of seven values preceding a drink (0, +5, +7.5, +10, +20, +35, and +50 minutes) was employed as the covariate. Single comparisons employed t-tests, paired within age groups or unpaired between age groups.

RESULTS

Maintenance of Euglycemia (Table I)

Basal blood glucose was greater in the old (91.8 ± 1.2 mg/dl) than in the young (84.3 ± 1.1 mg/dl) ($p < 0.001$). Euglycemia was well maintained following the glucose drink. Coefficient of variation for glucose (0–180 min) was 5.9% and 8.3% for the control and glucose drinks respectively in the young, and 4.7% and 6.4% for the control and glucose drink studies in the old.

Effect of Drink on Insulin (Table I)

Basal levels of serum insulin were 8.3 ± 0.9 uU/ml and 10.6 ± 0.6 uU/ml in the young and old groups respectively (p = NS). The 385–425 uU/ml range of levels achieved with the 120 mU/m² • min insulin infusion over the 60–180 min period was comparable to our previous work (Rowe *et al.*, 1983). The type of drink had no effect on the insulin levels achieved post-drink in either age group (p = NS by ANOVA).

Glucose disposal Rate (Table I)

In both age groups the total body glucose disposal rate achieved in the 60–180 min period after the control drink were comparable to the results of previous euglycemic clamp studies without drinks in our laboratory (Defronzo, 1979; Rowe *et al.*, 1983). The lower glucose disposal rate of 8.76 ± 0.3 mg/kg • min in the old compared to 10.09 ± 0.2 mg/kg • min in the young ($p < 0.01$) is consistent with age-associated differences in sensitivity of peripheral tissues to insulin (Rowe, 1983; Fink *et al.*, 1983). Due to absorbtion of oral glucose from the gut during the glucose study intravenous glucose infusion rates necessary to retain euglycemia were less than during control studies, ($p < 0.001$ for young and old).

Norepinephrine Response (Table II)

Basal NE was much greater in the old compared to the young ($p < 0.0001$). Plasma NE levels rose similarly in both study conditions in the young, consistent with the previously documented SNS response to hyperinsulinemia (Rowe *et al.*, 1981). NE increased 54% above basal to a peak of 284 ± 48 pg/ml at 150 min in the control study ($p < 0.006$), and increased 39% above basal to a peak of 241 ± 43 pg/ml at 180 min after the glucose drink ($p < .0005$). Plasma NE did not vary during the control study in the old (p = NS), consistent with a lack of SNS response to hyperinsulinemia previously reported to the old (Minaker, 1982). During the glucose drink studies in the old however, NE increased 42% above basal to a peak of 650 ± 87 pg/ml at 120 min ($p < 0.001$). This change in NE after glucose drink compared to control drink was statistically significant in the old subjects ($p < 0.01$) but not the young (Fig. 2). The difference in NE between the glucose and con-

TABLE I. RESULTS OF EUGLYCEMIC CLAMP STUDIES. ALL VALUES ARE MEAN ± SE

	Basal Glucose† (mg/dl)	*Basal Insulin†* (uU/ml)	*Mean Insulin Levels 60–180 min* (uU/ml)	*Intravenous Glucose Infusion to Maintain Euglycemia 60–180 min* (mg/kg • min)
YOUNG (n = 6)				
Control Drink			395.0 ± 28	10.09 ± 0.2
	84.3 ± 1.1	8.3 ± 0.9		
Glucose Drink			387.3 ± 35	5.38 ± 0.5
OLD (n = 8)				
Control Drink			420.9 ± 24	8.76 ± 0.3††
	91.8 ± 1.2*	10.6 ± 1.6		
Glucose Drink			420.5 ± 23	4.87 ± 0.3

†mean of −15 and 0 min values from both studies.
*$p < 0.001$ compared to young.
††$p < 0.01$ compared to young.

trol drinks was greater in the old group than in the young group over the 60–180 min period ($p < 0.02$) (Fig. 3).

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In the young, pulse rate ($p < 0.02$) and double product ($p < 0.02$) increased more after glucose than after control drink, despite no differential increase in NE. These differences were present immediately after the drinks and did not vary over time (drink-time interaction, p = NS). Pulse pressure and mean arterial BP were unaffected by type of drink. In

TABLE II. EFFECT OF CONTROL AND GLUCOSE DRINKS ON PLASMA NOREPINEPHRINE IN YOUNG AND OLD SUBJECTS*‡

	Basal†	*+50*	*+60*	*+70*	*+80*	*+90*	*+120*	*+150*	*+180*	*F§*	*p*
YOUNG (n = 6)											
Control Drink	185	206	213	209	222	208	251	284	245	3.29	<0.006
	±19	±25	±27	±14	±32	±16	±37	±48	±30		
Glucose Drink	174	176	200	196	216	230	225	219	241	3.39	<0.005
	±33	±21	±27	±34	±27	±37	±33	±33	±43		
OLD (n = 8)											
Control Drink	434	486	546	497	545	538	536	567	514	1.97	NS
	±35	±43	±56	±46	±50	±59	±25	±67	±45		
Glucose Drink	460	485	588	533	558	560	650	635	622	3.96	<0.001
	±39	±58	±100	±82	±43	±38	±87	±76	±82		

*All values are mean ± SE in pg/ml.
‡Drinks taken between 60 and 65 minutes.
†Mean of −15 and 0 minute values.
§Comparisons over time within a given study of ANOVA.

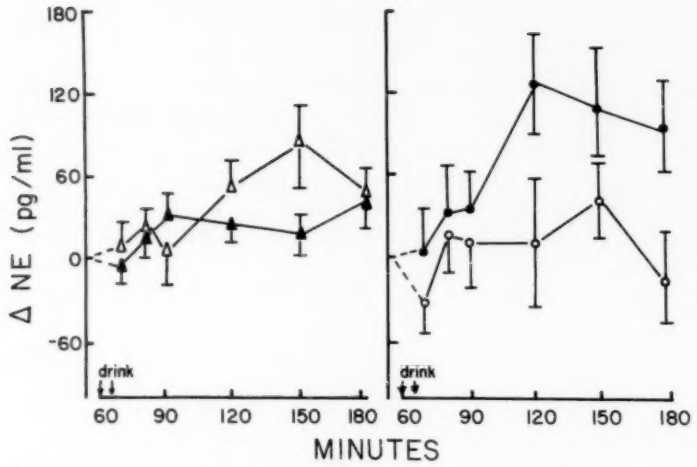

FIG. 2. Changes in plasma NE after glucose (▲) and control (△) drinks in the young (left panel), and after glucose (●) and control (○) drinks in the old (right panel). The change in NE after glucose compared to control is significant only in the old subject ($p < 0.01$) Points are mean ± SE.

the old group in which NE increased after the glucose drink, pulse rate ($p < 0.03$) was also greater after glucose drink compared to control. The difference varied over time (drink-time interaction $p < 0.04$). Mean arterial BP showed a nonsignificant trend of decreasing more after glucose than after control drink ($p = 0.11$). Pulse pressure and double product were unaffected by type of drink. When age groups were compared, there were no statistical differences in cardiovascular responses.

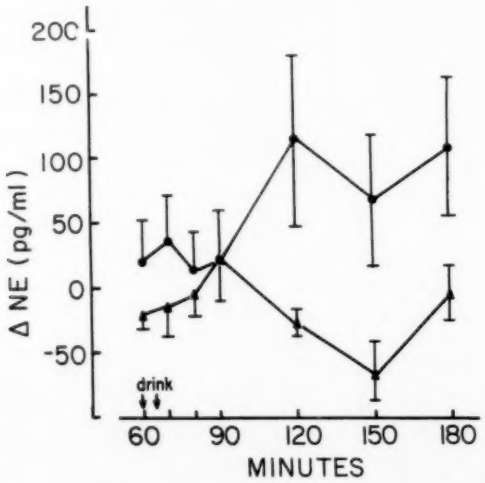

FIG. 3. Effect of oral glucose on plasma NE in young (▲) and old (●) during euglycemia. Data plotted represent the difference between plasma NE levels in paired glucose and control drink studies. There is significant difference between old and young ($p < 0.02$). Points are mean ± SE.

DISCUSSION

The present studies indicate that splanchnic factors play a role in the increased NE response to oral glucose in the elderly. Oral glucose ingestion raised NE levels during steady-state hyperinsulinemia and euglycemia. This differed from a noncaloric control drink of equal volume which had no effect under comparable conditions, consistent with resistance to an effect of hyperinsulinemia alone in this age group (Minaker *et al.*, 1982). In the young group, NE increased similarly following glucose and control drinks during hyperinsulemia and englycemia as had been previously observed (Rowe *et al.*, 1981). The elderly subjects, compared to the young for the effect of oral glucose, were found to have a significantly greater NE response.

The pattern of NE elevation in the old after glucose ingestion during hyperinsulinemia and euglycemia was similarly greater in the second hour. In our initial studies (Young *et al.*, 1979), NE was elevated in the second and third hours after oral glucose in the elderly. The pattern is not due to decreased clearance of NE with age (Young *et al.*, 1979). Others have supported this finding (Featherstone *et al.*, 1984; Hoeldtke and Cilmi, 1984; Rubin *et al.*, 1982), although a modest age-related decline in NE clearance has been reported in one study (Esler *et al.*, 1981). Whether the clearance of norepinephrine is affected by oral glucose as a function of age has not been explored. If such a mechanism were operating, it would be considered a splanchnic effect consistent with the conclusions of this study.

Cardiovascular parameters of pulse rate and double product in the young did increase to a greater degree with the glucose drink than with the control drink, but the difference was independent of time. Because plasma NE is only an indirect measure of the activity of the sympathetic nervous system, these cardiovascular changes without a measurable differential rise in NE may indeed reflect some SNS stimulation after glucose in the young. They may also reflect a nonspecific response to ingestion of nutrients as comparable changes in pulse rate have been noted after fat and protein ingestion in young subjects (Welle *et al.*, 1981). Pulse rate increased only after glucose drink in the old, temporally associated with the rise in NE. The comparable magnitude of these cardiovascular changes in young and old despite differences in measurable plasma NE are consistent with a diminished end organ responsiveness of cardiovascular tissues to SNS activation in the old (Lakatta *et al.*, 1975; Schocken and Roth, 1977; Vestal *et al.*, 1979).

The mechanism of increased NE after the glucose drink in the old is unknown. The trend of mean arterial BP to fall after glucose ingestion could be a factor, but this does not appear to be a causative factor. A comparable fall in mean arterial BP in previous 200 $mU/m^2 \cdot$ min insulin infusion studies was unassociated with increases in plasma NE in the old (Minaker *et al.*, 1982). Additionally, the time course of the rise in NE was established between 90 and 120 min in the glucose drink studies, a period in which mean arterial BP was stable.

Other potential factors involved in the increased NE after oral glucose in the old include vascular, osmotic, neural or humoral effects in the splanchnic bed (Young and Landsberg, 1979). The splanchnic vascular bed receives about 30% of the cardiac output at rest and may play a major role in our present findings. Age-related changes in gut function are controversial, particularly regarding differences in absorption and blood flow (Mayerson, 1982; Texter, 1982). The possibility of increased norepinephrine release stimulated by blood pooling in the splanchnic bed and subsequent modest reduction in right atrial pressure cannot be excluded (Johnson *et al.*, 1974).

The effect of nutrients and osmotic loads on plasma NE is not certain. During the pres-

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ent studies systemic osmolarity is held constant, so any osmotic factor would have to operate at a local level. Protein and fat ingestion which provide different osmotic loads than glucose have not been specifically tested in the old, but they have not shown any effect on NE in the young (Welle *et al.*, 1981; Young *et al.*, 1980). In these young subjects, oral glucose alone was associated with modest increases in NE compared to water ingestion, a result which can be understood as mediated by hyperinsulinemia in the young age group. The specificity of this effect has been questioned by the finding in other young subjects that oral glucose, xylose, mannitol and water equally raise plasma NE within 5–15 min of ingestion (Tse *et al.*, 1983). Local mechanical factors such as deglutination or gut distension, it was postulated, nonspecifically stimulate a sympathetic neural reflex that results in NE release. These mechanical factors appear to be adequately controlled in the present study by using an equal volume of water as the control drink. The present results support an effect of the glucose compared to a noncaloric water drink in the old.

Neural and humoral factors have been hypothesized to act at the level of the hypothalamus or brainstem to alter sympathetic outflow. Insulin and insulin-induced glucose metabolism within the central nervous system cannot account for the present effect of glucose in the elderly because the insulin and glucose levels achieved were equal in both drinks in the present study, and in previous work NE was not influenced by increasing levels of hyperinsulinemia and glucose disposal in the old (Minaker *et al.*, 1982). Some enteropancreatic polypeptides have different responses to oral glucose as a function of age, but their potential role in increasing NE is uncertain at this time (McConnell *et al.*, 1983; Rayford and Texter, 1983).

The present observations extend the data on the complex changes in sympathetic nervous system activity and effectiveness during aging. Enhanced splanchnic stimulated NE increases following oral glucose may reflect a hypersensitive response from a state of increased sympathetic nervous system activity in the old (suggested by the higher basal NE levels). It has been hypothesized that this state may result from a reset of central SNS outflow due to decreased sensitivity of the baroreceptor reflex with age (Pfeifer *et al.*, 1983; Rowe and Troen, 1980). It is clear that increased NE after oral glucose could further impair glucose tolerance in the aged. SNS activity is generally held to enhance hepatic glucose output by stimulating gluconeogenesis and glycogenolysis, suppressing insulin release, stimulating glucagon release from the pancreas, as well as reducing peripheral disposal of glucose (Young and Landsberg, 1979).

Both insulin and splanchnic factors appear to be involved in SNS stimulation after oral glucose. Insulin appears to exert a direct effect in the young, but not in the old who are known to have decreased insulin sensitivity. Splanchnic factors appears to predominate in the old.

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AGE RELATED CHANGES IN VISUAL ACUITY

NEIL S. GITTINGS and JAMES L. FOZARD

Gerontology Research Center, National Institute On Aging Baltimore, Maryland

Abstract—Longitudinal visual acuity assessments of men, and cross-sectional assessments of men and women in the Baltimore Longitudinal Study of Aging are presented. The longitudinal data relate presenting far, uncorrected far, presenting near and uncorrected near visual acuities to age. The cross-sectional data relate presenting far acuity to age. The prevalence of cataract, glaucoma and retinal pathologies are reported for the longitudinal sample at the time of their last vision test. The effect of visual pathologies in general, and cataract in particular, upon presenting far visual acuity was examined. The longitudinal data are consistent with cross-sectional data from previously published reports. Older persons who were free from specific visual pathologies exhibited an age-related decline in presenting far acuity as did those with documented visual pathologies. Despite the demonstrated loss in acuity with age, the majority of persons maintain at least fair acuity (20/40 or better) into their 80's.

Key Words: aging, visual acuity

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THE PRESENT paper describes longitudinal data on visual acuity collected in the Baltimore Longitudinal Study of Aging (BLSA) over a 21-year period. The report is timely for this special issue, for it is the first description of the longitudinal findings in visual acuity using the research plan devised by Nathan W. Shock.

Longitudinal evaluations of visual acuity have been reported by Anderson and Palmore (1974) using groups that were initially 60–69 and 70–80 years of age. Ten-year changes in acuity were reported for the better eye, with correction if any, and indicated increases of 13 and 32% in the percentage of the groups with 20/50 acuity or worse for the younger and older groups, respectively. Increases in the percentages with cataract, glaucoma and cornea guttata, also occurred. Milne (1979) used persons initially aged 63–90 years who were remeasured after one and five year intervals. Over the five-year interval, 12% of men and 14% of women exhibited worsened visual acuity. However, Milne also observed improvement in visual acuity in 15% of men and 10% of women. The prevalence of cataract in the sample also increased over five years from 15% in both men and women to 40% in men and 46% in women. The observed decline in acuity was associated both with age and the presence of cataract. Neither of the two studies provides information on acuity

Correspondence to: James L. Fozard, Ph.D., Associate Scientific Director, BLSA, NIA, Gerontology Research Center, 4940 Eastern Avenue, Baltimore, MD 21224.
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changes throughout the younger adult years, particularly as they relate to the level of acuity in earlier years. The present study provides such information.

Published results of cross-sectional studies of age differences in visual acuity provide comparisons to BLSA data that are useful in two ways. First, the BLSA participants represent a select group which may not be representative of the general population. Comparison with cross-sectional results will place the BLSA data in proper perspective relative to data from other representative samples. Second, results of longitudinal studies can complement those of cross-sectional designs with respect to specifying the antecedents of the observed cross-sectional results.

The target sizes employed in the present study correspond to nine Snellen fractions. To facilitate comparison of the data to other published findings, the acuity scores associated with each target level were converted from Snellen fractions to Snellen decimals, the decimal equivalent of the fraction (The decimal equivalent of 20/20 is 1.0, while that of 20/200 is 0.1.). The use of the Snellen decimal facilitates the graphic presentation of the serial observations reported; but placement of the Snellen decimals on an acuity scale is somewhat arbitrary inasmuch as the underlying scale is ordinal, nonlinear and without defined end points.

Noting that Snellen fractions can be represented on a logarithmic scale with 20/20 corresponding to zero, Weale (1978) presented a summary of cross-sectional studies of visual acuity and age using the logarithm of the Snellen decimal, while Pitts (1982) in a similar endeavor, employed Snellen decimals only. We have adopted the second, more frequently used convention, recognizing the limitations of each method. It should be clear that neither strategy alters the fact that successive rows of targets on vision charts do not increase in equal increments.

Weale's summary of cross-sectional studies of visual acuity and age, indicated that acuity is stable from about age 20 to 45. Loss of acuity begins at about age 45 and continues thereafter at a generally constant rate. Pitts' recent summary of age related decline in acuity incorporated additional cross-sectional data that provide confirmation of the pattern described by Weale.

The age related loss in accommodative power has been documented by Bruckner (1967). Although loss of accommodation is linear and proceeds over the lifespan, it becomes apparent to individuals during the age period from the late thirties to the mid-forties. Because of lost accommodative power, uncorrected near acuity declines abruptly between the ages of 40 and 45 and continues to decline thereafter at a slower rate.

The normal decline in the amount of light reaching the retina and the increase in the prevalence of certain visual pathologies are the most likely explanations for the changes that are observed in distance acuity with aging. Changes in the ocular system (such as senile miosis and opacification of the lens) cause the decline in the amount of light reaching the retina of the elderly. Cataract, glaucoma and various retinal disorders become more prevalent with advancing age. The decrease in the flexibility of the lens within the lens capsule is the main reason for the decline in accommodation that occurs with age. The present study investigates the impact that some of the aforementioned ocular changes have upon visual acuity.

METHODS

Data were obtained from male and female participants of the BLSA of the National Institute on Aging. The BLSA was initiated in 1958 in an effort to analyze physiological

and behavioral changes that occur with age and to determine the character of their relationships with one another. BLSA participants are atypical in that their socioeconomic characteristics "are those of an upper-middle-class segment of the general population" (Shock *et al.*, 1984). The typical interval between successive visits in the BLSA is two years. Over the observation period, there was a time frame during which participants over 70 were reexamined every year and those 60–69 every 18 months. As a result, the span of time represented by a given number of successive visits is not the same for all cohorts.

Vision tests were administered with a Titmus Optical Vision Tester, a stereoscopic instrument designed for rapid measurement of visual performance. The distance tests were presented at an optical distance of 20 ft, the near tests at 14 in. The brightness of the target slide was between 10 and 15 foot-lamberts, diffused to give uniform illumination to the entire target area. Right and left monocular acuities were determined with Sloan letters. Individuals were first tested without correction and then with their available corrective lenses if they wore them. The testing levels were: 20/200, 20/100, 20/70, 20/50, 20/40, 20/30, 20/25, 20/20, and 20/15 for distance vision; and 14/140, 14/70, 14/49, 14/35, 14/28, 14/21, 14/17.5, 14/14 and 14/10.5 for near vision. Visual acuity was defined as the acuity level which corresponded to the line preceding the one in which two or more errors occurred. One or more errors at low acuity levels, i.e. 20/200, 20/100 and 20/70; and 14/140, 14/70, and 14/49, dictated that the acuity be recorded as that of the preceding acuity level due to the small number of target letters at each level, one, two, and four respectively. Acuities which were worse than 20/200 or 14/140 were assigned values of 20/400 and 14/280, respectively.

Acuities are reported for the better eye. Both acuity without correction and presenting acuity are reported. Presenting visual acuities are defined as acuities either without correction for those participants who do not normally use corrective lenses or visual acuity with correction for those who wear corrective lenses.

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Data regarding visual pathology in the BLSA sample were gathered from diagnosis lists that are generated for each person at each visit. The typical source of diagnoses of visual pathology is a medical history. Diagnoses can be made or modified as a result of a physical exam performed each visit by a nonophthalmologist physician. Such ocular exams are done without pharmacologic mydriasis or cycloplegia. Objective determinations of refractive status are beyond the scope of the BLSA.

RESULTS

Table 1 presents characteristics of the sample from which the longitudinal data have been collected. This group is comprised entirely of 577 men, since women were only added to BLSA in 1978 and generally do not have sufficient visits for a longitudinal analysis. The mean ages of participants, grouped by five-year birth cohorts, are shown in the third column of Table 1.

Mean acuities on seven successive visits of each cohort are joined by a line and plotted against age for presenting distance acuity, uncorrected distance acuity, presenting near acuity and uncorrected near acuity in Figs. 1 and 2. The typical mean interval between successive tests for each cohort is two years with variations as shown in Fig. 1 and 2. The percentages of persons using corrective lenses at their last visit are 94, 96, 98, 100, 100, 98, 99, 98, 88, 73, and 66 for successively later cohorts with mean birthdates from 1895 to 1945.

Presenting distance acuity exhibits very little change until about age 60 when acuity

TABLE 1. SAMPLE CHARACTERISTICS.

Cohort	N	Mean Age	Mean Birthdate	Mean No. of Visits	Mean Visit Date
1895	34	78.4	1895.1	7.7	1973.5
1900	54	74.7	1899.9	8.2	1974.5
1905	48	69.5	1905.2	7.2	1974.6
1910	58	65.0	1910.1	8.3	1975.2
1915	70	60.4	1914.8	7.4	1975.1
1920	67	55.9	1919.7	7.3	1975.7
1925	78	50.7	1924.7	7.2	1975.4
1930	42	46.4	1929.8	6.2	1976.2
1935	35	41.7	1935.0	6.3	1976.7
1940	33	37.0	1940.1	5.5	1977.1
1945	58	33.7	1944.6	5.1	1978.3

Mean age and mean visit date represent means of each individual's mean age and visit date during the course of the study.

begins to decline. Uncorrected distance acuity, on the other hand, appears to decline in a linear fashion from age 30 to 80. The efficacy of therapeutic intervention is generally consistent over age, the mean difference between uncorrected and presenting acuity being +0.34.

The decline in presenting near acuity is also linear for the most part until about age 70. As expected, uncorrected near acuity displays a dramatic decline between the ages of 40

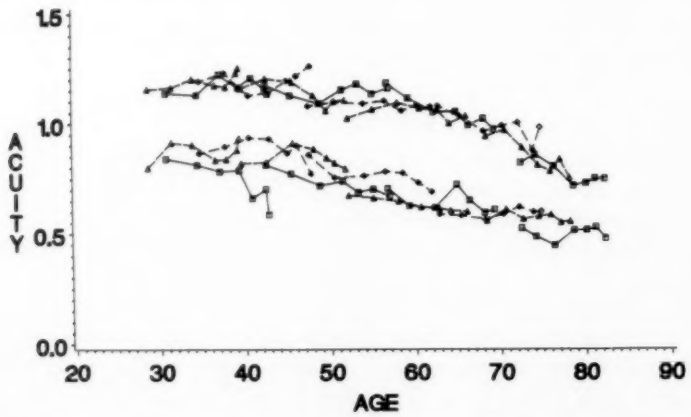

FIG. 1. Mean acuities (Snellen Decimal) on seven successive visits for 11 male cohorts. The upper plot represents presenting distance acuity; the lower, uncorrected distance acuity. Mean standard errors are 0.04 for presenting acuity and 0.07 for uncorrected acuity.

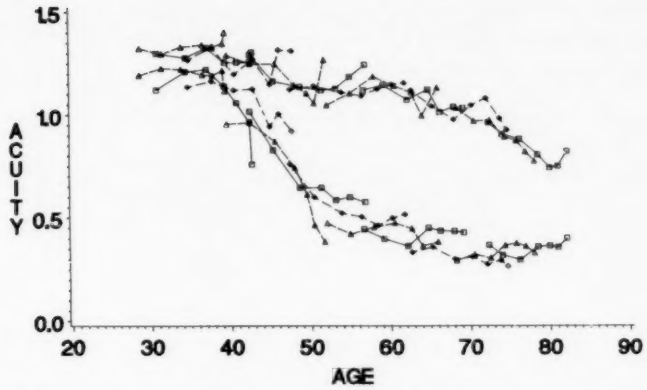

FIG. 2. Mean acuities (Snellen Decimal) on seven successive visits for 11 male cohorts. The upper plot represents presenting near acuity; the lower, uncorrected near acuity. Mean standard errors are 0.05 for presenting acuity and 0.06 for uncorrected acuity.

and 50, after which the decline lessens. Unlike distance vision, the degree to which corrective lenses or other therapies (e.g. surgery) are able to raise levels of acuity is not uniform over the years studied. Very little correction is needed between 30 and 40 years. Between 40 and 60 years, the amount of correction necessary and possible, as measured by the difference between uncorrected and presenting acuity, increases from about +0.20 to about +0.70. After 60 however, the improvement afforded by prescription lenses or other therapies decreases inasmuch as presenting acuity continues its decline.

It is interesting to note that although uncorrected near acuities are worse than uncorrected distance acuities after age 40, presenting acuities for near vision are consistently better than those for distance vision.

In terms of variability, the mean standard errors of the means on successive visits for presenting distance, uncorrected distance, presenting near and uncorrected near acuities are 0.04, 0.07, 0.05 and 0.06, respectively. For both presenting acuities, the variability was constant across all ages. For uncorrected distance and near acuities, however, there was a tendency for the standard error to decrease with advancing age from 0.11 and 0.09, in the youngest group, to 0.06 and 0.05, in the oldest group.

For purposes of statistical analysis, mean acuities were calculated over successive visits for each of the 577 participants. In addition, the change in visual acuity over successive visits was estimated for each of the participants using a linear regression procedure. The resulting mean acuities and slope estimates were used in the statistical analyses summarized in Table 2. One-way analyses of variance were performed on mean acuities and mean slopes of each acuity. The patterns suggested by Figs. 1 and 2 are corroborated by these data.

The longitudinal findings indicated that the amount and direction of change in acuity from first to last data point was related to the initial level of acuity. Persons with better vision at their first visit tended to change the most on subsequent visits. For those with good presenting distance acuity (20/15 and 20/20) initially, the average change from first to last visit is −0.15; for those with fair acuity (20/25 to 20/40) initially, +0.15; and for those with poor acuity (worse than 20/50) initially, +0.32 ($F = 50.79$, $p < 0.05$).

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TABLE 2. MEAN ACUITIES AND MEAN SLOPES.

	Distance				Near			
	Presenting		Uncorrected		Presenting		Uncorrected	
Cohort/Age	Acuity	Slope	Acuity	Slope	Acuity	Slope	Acuity	Slope
1895/78.4	0.76	-0.03	0.51	-0.01	0.81	-0.03	0.35	0.00
1900/74.7	0.85	-0.03	0.55	-0.02	0.90	-0.04	0.34	0.00
1905/69.5	0.97	-0.02	0.57	0.00	1.02	0.00	0.32	0.00
1910/65.0	1.03	-0.01	0.62	-0.01	1.07	-0.01	0.40	0.00
1915/60.4	1.04	0.00	0.61	-0.01	1.10	0.00	0.42	0.00
1920/55.9	1.07	0.00	0.75	-0.01	1.12	0.01	0.55	-0.02
1925/50.7	1.13	0.00	0.70	-0.01	1.17	0.00	0.66	-0.04
1930/46.4	1.15	0.00	0.81	-0.01	1.22	-0.01	0.75	-0.04
1935/41.7	1.18	0.00	0.82	-0.01	1.24	-0.01	1.03	-0.03
1940/37.0	1.15	0.00	0.74	-0.01	1.26	0.00	1.06	-0.01
1945/33.7	1.18	0.00	0.80	-0.01	1.33	0.00	1.14	0.00
F	22.78	5.98	3.72	0.88	26.15	7.18	40.63	10.21
	p<.05	p<.05	p<.05	N.S.	p<.05	p<.05	p<.05	p<.05

In order to assess any effect that cohort may have upon observed acuities, one-way analyses of variance were performed using first visit data. Each analysis used only persons who could be placed in a five-year age group based on their age at first visit. In effect, age was held constant within five years for each analysis. The dependent variable was presenting distance acuity; the classifying variable, cohort. Each analysis took into consideration 2 to 5 cohorts. Of seven one-way analyses, only one indicated a significant effect of cohort upon presenting distance acuity ($F = 6.97$, $p < 0.05$). The cohorts which produced the significant effect were 55 at first visit. The variability among cohorts at that age is evident in the upper plot of Fig. 1.

Utilizing a method similar to Arenberg's (1982), regression analyses were performed to investigate any effect of time of measurement upon presenting distance acuity. Only first visit data were included for analysis. Persons were assigned to one of 11 five-year age groups according to age at first visit. Separate slopes estimating differences in presenting distance acuity over calendar time were calculated for each group. If there were a systematic effect of time of measurement, independent of age, a nonzero slope relating date of measurement to acuity would be expected. None of the eleven slopes relating time of measurement to acuity differed significantly from zero.

In order to compare these data to those of other studies and to look at sex differences in acuity changes with age, we have plotted cross-sectional data from this sample and an additional sample of BLSA women on the composite plot compiled by Pitts (Fig. 3). All data were from the person's most recent vision test at the BLSA. At their most recent visit, the

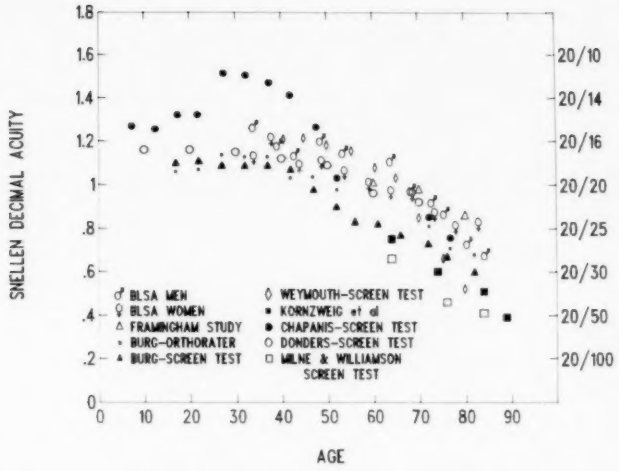

FIG. 3. Cross-sectional BLSA data added to Pitts' (1982) summary of age related differences in presenting distance acuity (Snellen Decimal). Data are from subjects' most recent visits.

male cohorts were 33.6, 38.8, 42.5, 48.1, 52.8, 58.3, 63.3, 67.6, 72.2, 75.1, 80.0, and 83.9 years old (One additional cohort born in 1950 has been added for this cross-sectional analysis). There are 27, 36, 27, 24, 26, 36, 42, 39, 51, 23, and 13 women in groups with mean ages of 33.4, 37.0, 43.1, 48.1, 53.1, 58.3, 63.3, 67.9, 72.7, 77.4, and 82.2 years, respectively. BLSA data are comparable to the other studies summarized by Pitts. There is a slight tendency for the older BLSA participants to be relatively closer to the top of the distribution. With the exception of the oldest women, acuities are slightly, but not significantly, better for men ($F = 3.62$, $p > .05$). This general finding held true for the other types of acuity as well.

The prevalence of visual pathology in the longitudinal sample of men is presented in Table 3. The percentages represent those who had a particular diagnosis appear in their diagnosis list by the time of their last vision test at the BLSA. The category, retinal pathologies, includes retinopathies as well as senile macular degeneration. Cataract and related groups are the most common visual pathologies in the BLSA (18%). The retinal pathologies classification is the second most reported pathology class (6%). Finally, about 3% of the sample are diagnosed as having glaucoma prior to their last vision test. As expected, the prevalence of cataract, retinal pathologies and glaucoma increases cross-sectionally with advancing age.

Figure 4 illustrates the effect of visual pathology upon presenting distance visual acuity by plotting a cross-sectional sample of acuities of persons who have no record of visual pathology with the remainder who have at least one diagnosis of visual pathology. Again, the diagnoses included in the morbidity group are cataract, aphakia, intraocular lens implant, glaucoma and retinal pathologies. Acuity data are from persons' most recent visits. In the seven oldest age groups (the 1925 cohort through the 1895 cohort), the percentages of each having at least one eye related diagnosis were; 12.8, 13.4, 31.4, 31.0, 54.2, 61.1, and 64.7, respectively. An analysis of variance performed on the means in Fig. 4 indicates that there are significant main effects of visual pathology ($F = 103.94$, $p < 0.05$) and age

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TABLE 3. PERCENTAGE OF BLSA PARTICIPANTS HAVING DIAGNOSES OF VISUAL PATHOLOGY WHICH APPEARED AT OR BEFORE THEIR LAST VISION TEST.

	Lens pathologies			Glaucoma	Retinal Pathologies
Cohort/Age	Cataract	Aphakia	IO Implant		
1895/78.4	41.2	8.8	2.9	5.9	8.8
1900/74.7	42.6	9.3	0	9.3	13.0
1905/69.5	35.4	8.3	0	6.3	10.4
1910/65.0	12.1	1.7	1.7	8.6	10.3
1915/60.4	14.3	2.9	2.9	4.3	7.1
1920/55.9	7.5	1.5	0	1.5	3.0
1925/50.7	7.7	1.3	0	0	5.1
1930/46.4	0	0	0	0	4.8
1935/41.7	0	0	0	0	2.9
1940/37.0	0	0	0	0	0
1945/33.7	0	0	0	0	0
Totals	82	17	4	19	35

($F = 13.41$, $p < 0.05$), but no significant interaction between visual pathology and age ($F = 0.65$, $p < 0.05$). The conclusions to be drawn from this analysis are that even in the absence of documented visual pathology, most persons experience an age-related decline in acuity independently of the effect of visual pathology.

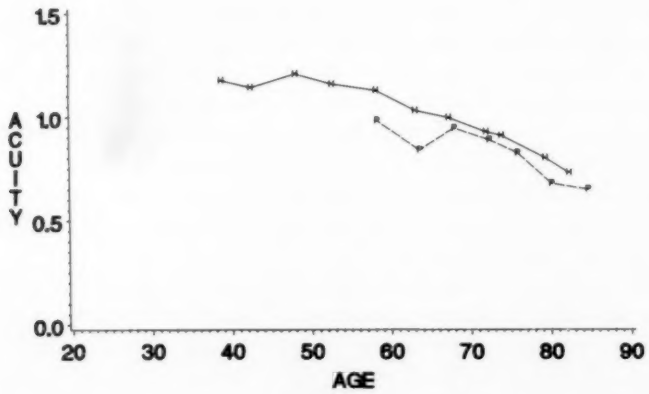

FIG. 4. Cross-sectional plots of presenting distance acuity (Snellen Decimal) obtained at the subjects' most recent visits. Subjects with apparently healthy eyes, represented by and "H", are compared with subjects, represented by a "P", who have at least one diagnosis of visual pathology. Mean standard errors are 0.04 for the healthy eye group and 0.06 for the group with ocular pathology.

TABLE 4. PRESENTING DISTANCE ACUITY FOR VARIOUS CATARACT GROUPS.

Cohort/Age		None	Present	Aphakic	Implant
1895/78.4	Mean	0.77	0.57	0.75	0.20
	N	16	14	3	1
1900/74.7	Mean	0.80	0.67	0.61	
	N	26	23	5	0
1905/69.5	Mean	0.87	0.79	1.04	
	N	27	17	4	0
1910/65.0	Mean	0.92	0.85	1.33	1.00
	N	49	7	1	1
1915/60.4	Mean	1.00	0.81	0.80	1.07
	N	56	10	2	2
1920/55.9	Mean	1.02	0.77	0.80	
	N	61	5	1	0
1925/50.7	Mean	1.12	0.91	1.33	
	N	71	6	1	0
1930/46.4	Mean	1.15			
	N	42	0	0	0
1935/41.7	Mean	1.21			
	N	35	0	0	0
1940/37.0	Mean	1.14			
	N	33	0	0	0
1945/33.7	Mean	1.18			
	N	58	0	0	0
Totals		474	82	17	4

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The particular effect of cataract status on presenting distance acuity is summarized in Table 4. Acuities are from the person's most recent visit. Presenting distance acuities for persons placed in one of the three lens related categories were obtained from eyes which had no documented history of cataract in 12 cases. Therefore, acuities for persons in the column headed "Present" were for the eye with the opacity, or in the case where both eyes had cataracts, the acuity of the better eye. The acuities in the columns headed "Aphakic" and "Implant" were for the enucleated eye. For the 1925 and earlier cohorts, the mean clear lens acuity is 0.98 and that of the eyes with lenticular opacities is 0.74 ($t = 6.46$, $p < 0.05$). In the same cohorts, acuity in aphakic eyes, mean of 0.85, is slightly better than that of cataractous eyes ($t = 1.27$, $p > 0.05$), but is not as good as that of the eyes with clear lenses ($t = 1.40$, $p > 0.05$). Two out of the four implant cases, mean acuity of 0.83, achieved acuities of 20/20 or better. The individual in the 1895 cohort with an acuity of 0.20 (20/100), had bilateral implant surgery in 1975, but has no other history of visual pathology.

DISCUSSION

The degree of agreement among the illustrated acuity data in Fig. 3 is remarkable when one considers the variation in the control of variables that are known to affect acuity. Very few, if any of the previous studies used exactly the same techniques for measuring acuity or controlling variables related to acuity, e.g. distance from the target, time of ex-

posure to the target, illumination of the target area, size of the target, contrast between the target and its background and refractive status of the eyes. In fact, this study, and the others referred to in Fig. 3, have focused on just two factors related to acuity, i.e. distance and size of target.

As indicated earlier, the scaling conventions used by various investigators have varied. To illustrate the problem with using Snellen decimals, a change in acuity from 20/20 to 20/40 converts to a difference in Snellen decimals of 0.5, whereas a change from 20/100 to 20/200 is equivalent to a difference of only 0.1. Using the logarithm of the acuities equalizes the separations on a scale between 20/20 and 20/40 and between 20/100 and 20/200. The mean logarithms of presenting distance acuity (men) from the cross-sectional BLSA data is 0.10 (Snellen Decimal equal to 1.27) for the youngest group and −0.20 (Snellen Decimal equal to 0.68) for the oldest group. Use of logarithmic scaling of these test values results in a 300% decrease in the logarithm of visual acuity. In contrast, without converting to a logarithmic scale, there is a 46% decrease. The choice of scaling does not change the fact of age related change in visual acuity, only the impression of its magnitude.

Anderson and Palmore observed ten-year increases of 13 and 32% in the percentage of two groups with 20/50 acuity or worse. The groups were initially 60–69 and 70–80 years of age. With BLSA data grouped in the same way as Anderson and Palmore's, we observed a decrease of 2% and an increase of 20% in the groups over a ten-year period. Using Milne's procedures and definitions, a group of BLSA participants initially aged 63–90 were reexamined after a five-year period. Six percent exhibited improved acuity while 18% experienced a decline in acuity as opposed to Milne's findings of 15% improved and 12% worsened. Our findings moreover indicate that those with improved acuity are likely to have had poor acuity initially. With some qualifications with respect to degree of change according to initial levels, the longitudinal data presented here confirm the patterns of change in acuity with age found in the previous longitudinal studies.

Weale (1961) has suggested that the light reaching the retina of a 60 year old is 1/3 that reaching a 20 year old's. Since the illumination of the target in this study was constant and low compared to environmental levels, older persons were at a disadvantage in terms of the amount of light reaching their retinas. If our illumination had been greater, there is a chance that we may not have observed as large a decline in acuity. Participants who are aphakic or who have intraocular implants should have more light reaching their retinas since clouded lenses have been removed. However, most of these persons have acuities similar to those at the same age without cataract, but not as good as those who are younger.

Cross-sectional comparisons of BLSA visual pathology data with those of Anderson and Palmore indicate that in the BLSA, the prevalence of lens pathology in groups over 60 ranges from 9 to over 50%, whereas Anderson and Palmore's observed rates range from 9 to 36%. In terms of glaucoma, BLSA prevalence in groups over 60 ranges from 2 to 9% as opposed to 0 to 3% in Anderson and Palmore's sample. A similar comparison is not possible for retinal pathologies. These statistics indicate that the prevalence rates of visual pathologies have not been underestimated in the BLSA.

There were 14 cases where a diagnosis appeared after the last vision test was performed. By definition these cases were assigned to the nonpathology groups, even though the individuals were probably in earlier stages of the disease at the time of their last vision assessment. Formal diagnosis of visual pathologies is somewhat dependent on factors unre-

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lated to the disease itself; for instance, time between visits to a physician. Pathology data in studies such as the BLSA is problematic in that we rely on participant reports based in part on information given them by their physicians. The information from our physical exam is not definitive. Accordingly, our prevalence data may be subject to greater error than would be the case with a focussed ophthalmological examination.

In conclusion, these longitudinal data confirm and document definitively the relationship between age and visual acuity suggested by cross-sectional studies. Older persons who are thought to be free from visual pathology still experience a decline in acuity with age as do persons who have some visual pathology. Despite the decline in acuity with age, only about 12% of the persons 75 and older had acuities of 20/50 or worse at their most recent visits to the BLSA. The majority of BLSA participants maintain at least fair acuity (20/40 or better) into their 80's.

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AGING OF THE LUNGS: SPECULATION ON THE ROLE OF OXIDANTS AND PROTEASES

CHARLES MITTMAN

Executive Medical Director, City of Hope Medical Center, Duarte, 1500 East Duarte Road, California 91010

Summary—In most regards, aging of the human lungs mimics the development of emphysema. Various lines of evidence suggest that oxidants and proteases play an important role in the pathogenesis of emphysema. The disease seems to arise through a complex interplay of genetic and environmental factors. This paper explores the possibility that normal aging of the lungs, as it has been defined by various cross-sectional and longitudinal studies, is part of a pathological continuum ending with frank emphysema. It is not clear if these are "normal" aging effects or the result of the accumulation of disease.

Key Words: lung, aging, oxidants, proteases, emphysema

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IN HUMANS it may be difficult to distinguish the effects of age on an organ system from the cumulative insults of disease. This is particularly true of the lungs. Ignoring the physiological consequences of stiffening of the chest cage, an age effect we demonstrated 20 years ago at the Gerontology Branch (Mittman *et al.*, 1966), the preponderance of studies indicate that aged lungs take on characteristics of emphysema. Little has been added to the literature on this topic which was summarized in a symposium published in 1964 (Cander and Moyer). In emphysema, alveolar walls are destroyed with consequent enlargement of alveoli and loss of the elastic properties of the lungs. Evidence that aging produces an emphysema-like state includes demonstrations of the loss of elastic recoil and enlargement of alveoli, increase in the resting volume (functional residual capacity) and the minimum expiratory volume (residual volume), as well as loss of vital capacity and reduction in forced expiratory flow rates. The lungs become less efficient in gas exchange; resting arterial oxygen saturation falls. Again, this change results from an emphysema-like defect, the progressive uneven distribution of ventilation in the lungs. This aging effect was demonstrated at the Gerontology Branch (Edelman *et al.*, 1968). In that paper we also reported that, in older subjects, large tidal volume breaths corrected this unevenness. Large breaths had no effect in the young. Holland, Milic-Emili, Macklem and Bates (1968) explained this observation, ascribing it to the enhanced tendency in the elderly for

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airways to collapse at low lung volumes. Large breaths open these collapsed airways, reversing the uneven ventilation. In older subjects, airways are more likely to collapse because of the lung's loss of elastic properties and the resultant retractive support of the airways.

Appreciation of the similarities between the changes in the lungs associated with aging and the defects in emphysema has not been translated into an understanding of the mechanisms underlying these aging changes. Indeed, it is only recently that we have gained some understanding of the biochemical basis for emphysema. I would like to propose that the rapidly evolving knowledge of the effects of oxidants and proteases on the lungs has not only helped explain the basis for the disease emphysema, but now offers an explanation for the observed age changes in lung function.

In the early 1960's two seemingly unrelated observations were reported from opposite sides of the Atlantic. These initiated an active field of research which has yielded a wealth of information about the lungs. Laurell and Eriksson (1963) were the first to report a series of patients from Sweden demonstrating the association between inherited severe α_1 protease inhibitor deficiency and the development of emphysema. Patients with the severe deficiency, who represent one out of approximately 2000 births in certain populations, carry two abnormal genes (labeled Z) and have approximately 10 to 15% of the normal level of the protease inhibitor which comprises the bulk of the α_1 globulin. The normal phenotype (PiM) is present in 95% or more of the population. Many other variants in this highly polymorphic system have been reported; they are rare and generally cause no deficiency. Alleles are codominant; carriers of the PiMZ phenotype are partially deficient. Most of those with the PiZ deficiency develop emphysema, particularly if they smoke: Typically, the disease is manifest by age 40 and the patient may be dead by 50.

In what then seemed to be an unrelated observation, Paul Gross (1965) reported that tracheal instillation of the protease papain produces an emphysema-like lesion in experimental animals. This observation has been confirmed repeatedly in studies using various experimental animals and different proteases. Emphasis is now given to the observations that elastase derived from white blood cells can produce the lesion. The digestive effect is upon lung connective tissue proteins, elastin probably being the most important. The effect is enhanced by inducing a deficiency of the protease inhibitor. The physiological consequences of these exposures are identical to the defects seen in emphysema.

These observations led to the promulgation of the "protease-antiprotease" theory of the development of emphysema. In this formulation, emphysema can result from damage to the lung's elastin network, caused either by an absence of the normal protective antiprotease or from excessive exposure to proteases. Initially only the rare patients with the PiZ phenotype were considered to be so affected. Data have now accumulated suggesting that the majority of patients with normal levels of the protease inhibitor suffer their lung injury through this or a very similar mechanism. Janoff (1985), who has contributed seminal observations to this field, recently summarized the topic. In brief, cigarette smoke and other agents implicated in the etiology of emphysema can increase the elastolytic attack on the lungs by initiating mechanisms that release leukocytic proteases in the lung interstitium. Concurrently, cigarette smoke and other extrinsic and intrinsic oxidants degrade the antiprotease in vitro and may produce a localized antiprotease deficiency at the site of elastin attack. Interest has focused on the fact that the same inflammatory reactions that can liberate excessive protease in lung tissue also produce oxidants. This work

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has been give greater importance by the imminent release of large supplies of the natural antiprotease obtained through blood fractionation and by the prospect for the availability of unlimited quantities of material produced by genetic engineering. Treatment for the PiZ patient has started. The place for augmentation therapy in patients without the deficiency is under discussion.

Having described this clearcut picture of the etiology of emphysema, it must be pointed out immediately that the picture is far more complex. For example, not all individuals with severe antiprotease deficiency develop emphysema. Rarely, emphysema may develop early in life with little cigarette exposure in the presence of normal amounts of the antiprotease. Thus, antiprotease deficiency does not explain all patients with an extreme susceptibility to the disease. Clearly there are also individuals who are protected from developing the problem, since we often encounter heavy smokers with no signs of illness even at an advanced age. Such clinical observations are consistent with the hypothesis that one's risk of developing emphysema is the result of a complex interplay over a lifetime of inherited or other intrinsic factors and environmental influences. Cigarette smoke is the most important environmental exposure, but industrial toxins and other agents can play an etiological role. Infections may be important, especially some occurring early in life. Antiprotease deficiency may be but the first of a series of inherited factors which will be recognized as playing a role in the equation determining if emphysema develops. We have made observations which suggest that a major component of the serum antioxidant system is under genetic control (Taylor *et al.*, 1983). This may be another in the list of controlling intrinsic factors which influence susceptibility to the development of emphysema. In patients with emphysema who have a defect in this antioxidant defense two possible hazards could be operating. First, the patient's antiprotease would be more susceptible to degradation. Further, oxidants could more easily attack the lung parenchyma. The latter effect, however, may not be important. The lesion typically resulting from oxidant injury to the lung is pulmonary fibrosis and not emphysema (Cantin and Crystal, 1985).

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If one's risk of developing emphysema is dependent on multiple factors, as described here, then it is not difficult to envision that the normal aging changes in the lungs described in the literature are an extension of that same equation. Subjects selected for normative aging studies tend not to have overt emphysema. Such patients are excluded from cross-sectional studies or the susceptible segment of a population are undersampled due to the survivor effect. Longitudinal studies tend to employ health conscious individuals who experience low smoking rates. Yet even in these biased populations, after the passage of enough time, there will have been significant exposures to environmental oxidants and other irritants. Episodes of inflammation of the lungs will have occurred in most individuals bringing leukocytic proteases and oxidants into the lung parenchyma. Anatomic observations support this notion. Auerbach *et al.* (1972) measured the degree of emphysema in a large series of autopsy specimens. The lesions increased in severity and extent with increasing cigarette exposure and with increasing age. However, the age gradient was minor in nonsmokers.

The early literature on aging speculated that oxidants might account for the changes seen in the cardiovascular system. It is not clear why investigators ignored this as an explanation for changes seen in the lungs. This would have been a small extension in logic, given the recognition even then that the lungs are constantly exposed to oxidants. Although not yet proven, evidence summarized here implicates oxidants and protease as the basis

for some of what has been described as the age changes in lung function. Yet to be resolved is the issue of the extent to which these changes represent a "normal" process as opposed to the cumulative effect of disease.

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SURVEY RESEARCH GUIDING PUBLIC POLICY MAKING IN MARYLAND: THE CASE OF ALZHEIMER'S DISEASE AND RELATED DISORDERS

WILLIAM REICHEL,

Department of Family Practice, Franklin Square Hospital, Baltimore, Maryland

MICHAEL S. FRANCH and JERRY SOLON

Governor's Task Force on Alzheimer's Disease and Related Disorders, State of Maryland

Abstract—The Maryland Governor's Task Force on Alzheimer's Disease and Related Disorders conducted a comprehensive 18-month assessment of the extent of dementing illness in Maryland, the needs of Maryland dementia victims and their families, and the availability of resources to meet these needs. A major tool in this assessment was a series of 16 specially designed surveys which were sent to physicians, family caregivers, nursing homes, geriatric aide training programs, and other populations. The survey findings, along with hearing testimony, provided the basis for the Task Force's 18 principal and 35 further recommendations, published in the *Maryland Report on Alzheimer's Disease and Related Disorders* (Governor's Task Force, 1985). These recommendations have become the basis of public policy discussion for services to dementia victims and their families in Maryland.

Key Words: Alzheimer's Disease, dementia, dementing illness, public policy, long-term care, aging, health care services, health care legislation.

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INTRODUCTION

NATHAN SHOCK's demonstration of conceptual and empirical breadth in pioneering the development of gerontology included public service in Maryland related to the care of the elderly. Honoring Nathan Shock who bridged scientific knowledge to public policy implementation, this report summarizes the efforts of the Maryland Task Force on Alzheimer's Disease and Related Disorders (Governor's Task Force, 1985).

While laboratory and clinical studies pursue the search for elusive diagnostic markers, underlying pathogenesis, and management approaches for Alzheimer's and related

Correspondence to: William Reichel, M.D., Chairman, Department of Family Practice, Franklin Square Hospital, 9000 Franklin Square Drive, Baltimore, MD 21237.
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dementias, public concern with the severity and magnitude of the problem is markedly mounting. Currently, fully one-half of the states are targeting official fact-finding and legislative efforts to this problem (Alzheimer's Disease and Related Disorders Association, 1986).

In this milieu of current high interest in dementing illness, even the most cloistered biochemists and neurophysiologists find themselves testifying before State or Federal legislators seeking wisdom on how to divide the limited health care dollar. They may even be summoned from their research laboratories for service on public bodies similar to that described in this article. Some may view such calls to public service as burdensome intrusions on research time, but they are also opportunities for the scientific community to use its knowledge to affect public policy. In Maryland, Governor Harry Hughes created a Task Force on Alzheimer's Disease and Related Disorders (ADRD) in January 1984, noting that with the population's aging, dementing illnesses are destined to become more common and to exact a mounting toll in personal and social costs. He instructed the Task Force to submit a report to him and the Maryland legislature within 18 months. The report, duly submitted on June 30, 1985, contained an assessment of the problem of dementing illness in the State and recommendations for dealing with it.

METHODOLOGY

The Task Force faced two broad tasks: first, to collect complete, accurate, and detailed information; and secondly, to formulate recommendations based on the data it collected. In determining what information to gather, and then in assessing it and making recommendations, the Task Force's first resource was its own knowledge and experience. The Task Force was a 16-member interdisciplinary team that brought its own considerable knowledge, experience, and judgment to the work. Members were highly knowledgeable in direct care, public services, education, community resources, the law, clinical practice, and medical research. They included four physicians (representing Family Practice, Internal Medicine, Neurology and Psychiatry), three nurses, two social workers, an insurance executive, a lawyer, and civil servants. The two consumer representatives, husbands of dementia victims, were invaluable to the Task Force's work, as were the two representatives of the Maryland General Assembly, who brought penetrating minds and political savvy to the project. The Task Force also brought in authorities in areas beyond its own expertise to contribute their particular knowledge.

As much information as the Task Force had available, it nevertheless felt compelled to undertake original information collection in order to assess confidently the adequacy of existing resources in services, education, finances, and data development. It generated the needed information through two principal processes:

(1) Public hearings, nine in all, were held throughout the state. A cross-section of expert witnesses, family caregivers, agency representatives, and community leaders gave testimony.

(2) A series of mail surveys was conducted, addressed to the 16 categories of resources listed in Table 1. (Additionally, awareness of the Task Force's existence prompted unsolicited letters from concerned citizens, family caregivers, and health care professionals). The target populations were variously covered by the questionnaires' mailings—to care facilities and organized agencies and programs, and to a random

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TABLE 1. SURVEY CATEGORIES AND RESPONSES.

	RESPONSE	
	Volume	Rate (%)
Health Facilities Association of Maryland	1	100
Insurance companies	11	79
Law enforcement agencies	11	79
Public Schools	19	79
Professional education and training institutions	32	71
Geriatric aide training programs	60	62
Hospitals	34	52
Day care centers	18	50
Paramedic training programs	22	47
Home health care agencies	41	39
Research investigators	6	35
Fire departments	9	33
Nursing homes	71	27
Caregivers	152	22
Public and private service agencies	129	19
Physicians	46	14
TOTAL RESPONSES	662	27

selection of physicians geographically stratified; caregivers addressed were those known to the Alzheimer's Disease and Related Disorders Association (ADRDA).

Survey data and hearing testimony are by their nature more qualitative than quantitative, conveying, as they do, largely judgmental and anecdotal content. In tapping these resources, the Task Force sought the insights stemming from experiental material, and the strands of consensus converging from the variety of informants.

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RESULTS

The surveys, public hearings, and study of previous research gave the Task Force a substantial basis on which to assess the extent of dementing illness in Maryland, project its probable increase, and evaluate how well existing services were meeting the needs of dementia victims and their caregivers.

Demography and Epidemiology of Dementing Illness in Maryland

The Task Force recognized that it would have to make public policy recommendations based on epidemiologically incomplete but nevertheless indicative evidence. The well-known difficulties of making accurate diagnosis of dementing illnesses (McKhann *et al.*, 1984) and the lack of death certificate data or a central registry of cases pose severe obstacles to estimating the number of dementia victims. In making its estimates and projections, the Task Force drew on population projections compiled by State planners (Maryland State Long-Term Care Planning Group, 1982; Maryland Department of State Planning, 1984) and epidemiologic studies of other communities (Brody, 1984). Fortunately, one of the few comprehensive studies of dementing illnesses in a large population focused on Baltimore (Folstein *et al.*, 1985). The Eastern Baltimore Mental Health Survey, part of the Epidemiologic Catchment Area Program of the National Institute of Mental Health, intensively studied three East Baltimore neighborhoods. This study, carried out by researchers of the Johns Hopkins School of Medicine, included clinical and laboratory workups of a sample of the neighborhood population. The study concluded

TABLE 2. PERCENT INCREASE IN AGED POPULATION, 1980–2000 DERIVED FROM MARYLAND DEPARTMENT OF STATE PLANNING, 1984.

	AGE 65–74		AGE 75–84		AGE 85+	
Year	Population	Growth in Numbers (% Increase)	Population	Growth in Numbers (% Increase)	Population	Growth in Numbers (% Increase)
1980	247,209	–	115,735	–	32,665	–
2000	332,581	85,372 (34%)	228,286	112,551 (97%)	86,461	53,796 (165%)

	TOTAL AGE 65+	
1980	395,609	–
2000	647,328	251,719 (64%)

that 6.1% of the population over age 65 had a dementing illness, with the overall rate increasing sharply with advancing age. These figures are quite comparable to those found by Brody and the Federal Task Force report on Alzheimer's disease (U.S. Department of Health and Human Services, 1984).

The State Planning Department's projections of the growth in the 65-plus population, combined with the generally accepted figures on the increasing incidence of ADRD in older age groups, provided sufficient data to enable estimates of the number of ADRD victims in Maryland today and project the numbers for given years in the future. Table 2 and Fig. 1 (both derived from Maryland Department of State Planning [1984]) demonstrate the projected increase in the aged population between 1980 and 2000. Based on a rough low estimate of 5% of these 400,000 citizens age 65 and over, there are about

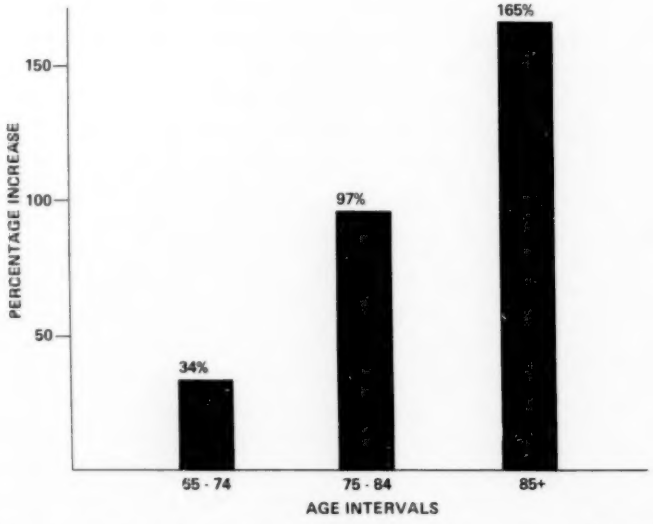

FIG. 1. Projected percentage increase in elderly population, 1980–2000 Derived from Maryland Department of State Planning, 1984.

20,000 Marylanders with Alzheimer's disease or a related disorder; by 1990 there will be 26,000, and by the year 2000 there will be more than 30,000 Marylandersd with ADRD—20,000 of them in institutions (mostly nursing homes) and another 10,000 being cared for in the community.

Availability of Resources

The core of the Task Force's assignment was to assess the adequacy of resources and to make recommendations. Resorting to the experiential reports from the whole spectrum of informant populations produced a remarkable mutual affirmation of key assessments of the state of affairs encountered by professionals, service agencies, and victims' families. A number of facets were revealed by this consensual process, relating to (1) information availability and education, (2) service gaps, (3) financial provisions, and (4) data needs.

Knowledge and Education. Many health care professionals and family caregivers lamented the lack of essential information about dementing illnesses, about types of resources, and about techniques for the care and management of dementia victims. The lack of needed information was sometimes revealed by "respondents" who returned a completely blank questionnaire with the explanation that they lacked the knowledge with which to reply to the queries. Many more respondents evidenced notable gaps of knowledge in their failure to respond to some important portions of the questionnaire. This lack of information may even have contributed to the low survey response rate from some quarters, if potential respondents felt inadequate to impart substantive information about resources. Many physicians, for example, frankly stated that they did not know what service and financial resources exist for dementia victims and families. Many family caregivers revealed themselves to be unaware of the existence of various listed types of services. (One troubled family caregiver lamented that "Only after a three-year search, I am just learning of resources available. Why didn't I know sooner?" An adult day care center reported: "Families don't even know what to ask for, and may go through a maze of blind alleys before help is obtained.") Indeed, the survey respondents amply indicate that there is a dearth of information in all sectors—families, providers, students, and educators. Some of the responses, such as rosy-hued declarations that services and resources for ADRD are generally available, apparently stem from simply being unaware and uninformed. But much of the gap in knowledge comes through explicitly, as from the physician who, in regard to availability of services, remarked that most of his answers "reflect my lack of knowledge."

The knowledge-gap is also revealed at times in a too-sharp discrepancy of responses among those in the same respondent category. Thus, among physicians there was a split regarding the adequacy of ADRD diagnostic services. The divergence of the aggregate responses that emerged respectively from the respondent categories of physicians and caregivers thus is all the more arresting:

- Although a majority (52%) of the responding physicians judged *diagnostic* services to be adequate, only one-third of the caregivers (32%) reported diagnostic services to be adequate.
- Physicians were evenly divided in their perception of the adequacy of *home health services.* By contrast, caregivers, by a ratio of 4-to-1 reported home health services to be inadequate.

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The implications of this discrepancy are serious. Obviously, if a respondent did not know of a service, that service effectively did not exist for that person and access to sometimes desperately needed relief is blocked. Conversely, physicians who think inadequate services are in fact adequate will be of little help in referral or as advocates for improvement in the system. Whatever the actual situation (the Task Force concluded that services are in fact generally inadequate), this is a disturbing difference in perception.

Survey respondents in all categories (physicians, family caregivers, paramedics, nursing home staff) expressed need for more information or education about dementing illness. In response to a query as to the adequacy of training programs for caregiver families, in the aggregate only one in ten respondents thought that provisions for such training were adequate. Some training programs for geriatric aides do not touch on dementia; but even those that offered such training appealed to the Task Force for assistance in enhancing their preparation, feeling their knowledge and efforts inadequate. Many respondents (including educators) criticized professional education programs for imparting insufficient knowledge and skills pertaining to ADRD. A school of nursing reported that it accords substantial coverage to Parkinson's disease and Huntington's disease, while "Alzheimer's disease is discussed but not in great depth." The educational system in medicine was targeted by statements such as "This entire area of medicine is badly neglected," and "Very few professionals know anything about ADRD."

Service Gaps. By far the strongest statements in the survey responses are sparked by concern with service. The overwhelming judgment is that existing services and resources are far short of being adequate. By a ratio of 3:1, respondents judged the existing provisions to be inadequate. Within this overarching judgment are important specifics:

Diagnostic services. As noted above, family caregivers and physicians sharply differed on the adequacy of diagnostic services.

Ongoing physician support. The respondent physicians had an even division among themselves as to whether or not physicians provide adequate ongoing support in ADRD cases. Comments from non-physicians reflect a rather pervasive feeling of lack of physician support in dealing with dementia patients.

Home health care. As noted above, physicians and family caregivers disagreed on the adequacy of home health care by, respectively, ratios of 1:1 and 4:1, the family caregivers preponderantly finding it inadequate. Caregivers made it very clear, in comments on the survey forms, testimony at hearings, and letters to the Task Force, that their lack of access to desperately needed home care resources is mainly rooted in financial capability for purchasing such service. As a home health agency pointed out, "No third party will pay for home health services, and that is what these patients need most."

Adult day care. Respondents have a very high regard for the value of adult day care for ADRD victims. It appears, however, that the available resources barely begin to meet the need. Thirteen of 24 counties in Maryland did not have any adult day care program at the time of the study. In the aggregate, respondents judged by a 2:1 ratio that adult day care provisions are inadequate. Insufficient transportation services in general, reported by a heavy majority in all respondent categories, are especially pinpointed by adult day care centers as posing a serious obstacle to potential users of their service.

Respite care, in-home. Clearly a highlight of the survey findings is the overwhelming judgment from all categories of respondents that in-home respite care is not adequately provided. Overall, those who report inadequacy of this service outnumber those who

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deem it adequate by 10:1. Among caregivers, the ratio catapults to over twice that magnitude. Here, too, the high cost becomes a barrier to a much-needed service.

Respite care, out-of-home. While not quite as much stressed as in-home care, there is, nevertheless, predominant judgment in all the respondent categories that out-of-home respite care needs to be increased. With concern both for the caregivers' health and for maintaining their capacity to continue to retain the patients in their homes, many deem it important that occasional respites of more than a few hours be obtainable.

Information and Referral Service. Much of the knowledge gap referred to above needs to be remedied by an information and referral service. Support for developing such a central resource was reflected by all respondent categories. Often singled out as a grievously neglected area of information is alerting of victims' families to certain critical legal provisions to attend to, to forestall crises.

Financial Concerns. In tandem with many of the concerns about availability of needed services are the financing aspects. "Spending down" into poverty is an ever-present anxiety for families with a demented member. The costs of engaging home care personnel, of enrolling the afflicted person in an adult day care program, or even of securing respite caregivers to relieve the family caregiver was frequently lamented as prohibitive on a continuing basis. Notorious are the restrictive eligibility provisions for long-term care under Medicare and Medicaid, and the absence or thinness of private insurance provisions. On questions as to adequacy of third-party coverages for ADRD sufferers, responses conveyed a dismal picture, except that physicians tended to a far rosier view of insurance availability than all other responding categories. The most tormented reports came from family caregivers.

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Data Needs. Planners, researchers, and executive and legislative policymakers are greatly hampered by lack of demographic and epidemiologic data about dementia victims. Gross estimates enable gross planning, but proper fine-tuning and targeting of provisions would be better guided by clear and dependable data about the afflicted, their circumstances and locations, and their care provisions and needs. In the course of its explorations, the Task Force perceived the need for expertise capable of developing more penetrating data than is now available.

DISCUSSION

It was on this base of findings that the Task Force made its recommendations to the Governor and General Assembly. Among a multitude of desirable proposals, the Task Force imposed on itself the task of prioritizing, in order not to swamp the system with an unrealistic overload of recommendations. In being selective of priority recommendations, the Task Force was guided by the twin considerations of (1) magnitude and urgency of need and (2) likelihood of implementation in the "real world," in the foreseeable future, including likely cost considerations. Yet, having identified many needed provisions, and acutely conscious that it would go out of existence with the submission of its report—and thus lose its platform for proposing additional recommendations—the Task Force presented two categories of recommendations. It highlighted 18 principal recommendations in the body of the Report, addressing those needs that were judged to meet the priority criteria. A second category of 35 recommendations, placed in an appendix entitled "Further recommendations," dealt with those needs which must be addressed in due time, but which the Task Force judged were not so pressing that they should detract from the

immediate implementation of recommendations which had a higher priority and were most readily achievable.

In keeping with the charge to the Task Force, its recommendations focused on Alzheimer's disease and related disorders. The Task Force believed, however, that many of the measures it proposed would be broadly applicable and could be tailored to benefit all types of chronically ill and disabled individuals. The Report states, "It is the hope of the Task Force that any measures and innovations described for the benefit of Alzheimer's disease and related disorders will also be applied more broadly and eventually will benefit other types of chronically ill and disabled individuals. The mandate of this Task Force, however, limits it to a specific category of disorders."

In the course of the Task Force's investigations, it became clear that the focus of improved services must be the family caregiver. What enables caregivers to continue caring for their loved ones, benefits the dementia victim. Reasons of compassion aside, the State has an interest in supporting family caregivers, as they frequently forestall institutionalization at State expense by maintaining the victim, at their own expense, in the home.

There is not space here for a detailed discussion of the Task Force's recommendations. (The entire report may be obtained, free of charge, from William Reichel, M.D., Franklin Square Hospital, 9000 Franklin Square Drive, Baltimore, Maryland, 21237.) The following is a very brief summary of the principal recommendations:

Summary of Principal Recommendations

Service Resources. Develop a comprehensive and coordinated system of service resources, including the following components: (1) develop a pool of respite caregivers to relieve family caregivers; (2) encourage and assist nursing homes and hospitals to be receptive to short-term respite stays by dementia patients; (3) expand and extend adult day care centers; (4) extend the network of mutual-help support groups; (5) provide legal and financial counseling through existing channels; (6) improve and extend diagnostic resources; (7) references in state documents to these entities should be amended so as not to be designated as principally "psychiatric disorders" since Alzheimer's disease and multi-infarct dementia are in fact organic disorders with secondary behavioral manifestations, which should be managed by an appropriate combination of medical, neurological, and psychiatric resources. (This seventh recommendation proved to be one of the Task Force's most controversial. Its genesis lay in the Task Force's concern that dementia victims and particularly their families suffered financially because insurance converage is generally inadequate for disorders labeled "psychiatric.")

Education and Training. Promote education and training, including the following actions: (1) develop dementia training centers of excellence; (2) incorporate dementia content in all professional education; (3) add dementia content to the State curriculum for geriatric aide training; (4) include dementia instruction in State-mandated inservice education by long-term care facilities; (5) engage the Alzheimer's Disease and Related Disorders Association—Baltimore/Central Maryland to conduct informational and educational activities, including a clearinghouse of informational materials, a statewide telephone hotline, and public education forums and professional continuing education programs.

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Financial Reliefs. Arrange financial reliefs, including the following: (1) extend the Gateway II program (assistance to cases who but for home care are at risk of early institutionalization) to all counties, and fund the program more fully; (2) seek easing of eligibility and service restrictions in Federal and State programs; (3) furnish financial incentives to families averting institutionalization costly to the State; (4) induce insurance industry to develop long-term care coverage.

Data. Develop statewide representative data on dementia, covering its extent, demographic distribution, and epidemiologic factors, and also on how services are organized and utilized.

Follow-through and Coordination. Charge a State agency with coordinating and monitoring progress on the recommendations of the Task Force on Alzheimer's Disease and Related Disorders. The Recommendations are more fully explained in the Task Force Report.

The Governor's Task Force on Alzheimer's Disease and Related Disorders provided a comprehensive survey of the needs of dementia victims and their families in Maryland. It is likely that the needs of Maryland ADRD victims and their families are similar to their counterparts in other states and that the recommendations of the Maryland Report apply to their needs as well. But while other jurisdictions may benefit from what task forces in Maryland or other states have learned, the process of investigation and assessment of each state should be undertaken. Although the needs may not be the same, the process of investigation and assessment is educational for both the experts and the lay public, and the publicity engendered by the process educates and sensitizes both the general public and the state legislators who must appropriate the money and change the regulations.

With shrinking budgets, growing elderly populations, and heightened competition for the health care dollar, it is incumbent upon policymakers to base their plans on accurate information. It is equally incumbent on busy researchers and clinicians to be willing to devote their expertise to such public purpose. Reasons of civic duty aside, there is the inherent reward of contributing to initiatives that may directly and beneficially bear on the lives of thousands of people. Within nine months of the release of the Maryland Report, the Maryland General Assembly enacted several of its recommendations into law. This record may not be so readily duplicated elsewhere—and much work remains to be done in Maryland—but it demonstrates accomplishment.

Acknowledgments—This article draws heavily from the Maryland Report on Alzheimer's Disease and Related Disorders. Carol Distasio, R.N., M.S., M.P.H., Research Consultant, played a major role in designing the survey forms. The Task Force members genuinely and vigorously threw themselves in the task, making massive investments of their time and energies. Task Force members were: The Hon. Rosalie S. Abrams, R.N., M.A.; Edmund Beacham, M.D.; The Hon. Clarence W. Blount, M.L.A.; The Hon. Peter G. Callas, Ed.D.; David F. Chavkin, J.D.; Jacqueline D. Fassett, L.C.S.W.; Fred M. Gloth, Jr., J.D.; Lloyd F. Hoover; Glenn Kirkland, M.S.; Patricia A. Lusk, R.N., M.P.H.; Nancy L. Mace, M.A.; Kay Mehlferber, R.N., M.A.; Patricia A. Newton, M.D., M.P.H., M.A.; Thomas R. Price, M.D.; and Valeria A. Tocci, L.C.S.W. Andrew Kalathas, J.D., of Blue Cross and Blue Shield of Maryland and Shirley Whitfield of the Maryland State Office on Aging served as liaison support. Special appreciation is expressed to Glenn Kirkland, M.S., for his analysis of data.

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EFFECTS OF AGE, HYPERTENSION HISTORY, AND NEUROTICISM ON HEALTH PERCEPTIONS

ALAN B. ZONDERMAN

Gerontology Research Center, National Institute on Aging, Baltimore, Maryland

VICTORIA L. LEU

Department of Medical Psychology, Uniformed Services University of the Health Sciences, Bethesda, Maryland

PAUL T. COSTA, JR.

Gerontology Research Center, National Institute on Aging, Baltimore, Maryland

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Abstract—The effects of measured blood pressure, history of hypertension diagnosis, age, and neuroticism on number of somatic complaints and self-rated health were examined in a sample of 970 non-health-care-seeking adult men and women. Significant differences in number of somatic complaints and self-rated health were found due to age, neuroticism, and history of hypertension diagnosis. Measured blood pressure, however, was unrelated to both measures of health perception. With the exception of the effect of neuroticism on somatic complaints, the effects of the independent variables on health perceptions were rather small in magnitude and explained only small proportions of the variance. Age differences had a particularly weak effect on health perceptions, accounting for less variance than either neuroticism or history of hypertension diagnosis. A significant interaction of neuroticism with awareness of hypertension was found, but only for number of somatic complaints. These results suggest that health perception is a complex, multidimensional construct. The relatively weak influence of hypertension diagnosis on health perception may account for the difficulties in maintaining patient compliance with antihypertensive treatment.

Key Words: age differences, awareness of hypertension, health perceptions, hypertension, neuroticism, psychological distress, self-rated health, somatic complaints

HYPERTENSION IS a leading health problem in the United States. Data from the Framingham Heart Study and the National Center for Health Statistics have documented elevations in systolic blood pressure with increasing age. Prevalence of hypertension similarly increases with aging so that hypertension has been estimated to affect as many as 50% of the elderly by their seventh decade. Not only is hypertension a major risk factor for coronary heart

Correspondence to: Alan B. Zonderman, Gerontology Research Center, National Institute on Aging, Francis Scott Key Medical Center, Baltimore, MD 21224.
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disease (Kannel, Gordon and Schwartz, 1971), but prospective studies have identified elevated blood pressure as a major risk factor for congestive heart failure, stroke, renal failure, and intermittent claudication. Antihypertensive treatment has been strongly associated with a reduction in the incidence of these complications further strengthening the evidence for elevated blood pressure as their primary determinant. Hypertension and its complications are thus common among the elderly, and its appropriate diagnosis and treatment is of major importance to their health.

Considerable effort has been devoted to detecting and treating hypertension and practitioners are particularly concerned about patient compliance with drug and diet therapies because of the asymptomatic nature of disease. Concern has arisen about the possible psychological effects of labeling patients with hypertensive diagnoses. Several studies have shown that hypertensives who are aware of their condition report more somatic symptoms than those who are unaware of their condition. These results suggest that labeling patients with hypertensive diagnoses may lead to psychological distress, symptom reporting, and poor self-rated health. These findings are unclear, however, because awareness of hypertension has been confounded with self-selection and few studies have examined the potential moderating role of personality dispositions such as neuroticism. The purpose of the present report is to clarify the relationships among history of awareness of hypertension, blood pressure, symptom reporting, and self-rated health. Specifically, we will examine the effects of measured blood pressure, history of hypertension diagnosis, neuroticism, and age on somatic complaints and self-rated health.

Somatic complaints as a function of age and neuroticism

Self-perceptions of health are of great importance because they influence many health-related behaviors such as seeking medical treatment and self-medication. Studies have repeatedly shown that global self-ratings, which are moderately correlated with medical determinations of health, are also related to psychological characteristics such as health attitudes, morale, adjustment and psychological maladjustment. In fact, general health attitudes and other psychological characteristics are more strongly associated with self-ratings of health than age, despite the increased frequency and severity of some conditions and diseases in the elderly.

Longitudinal studies have shown that age has a selective effect on somatic complaints. That only certain somatic systems show age-related increases was suggested as a possible explanation for why global health ratings are only weakly related to age. On the other hand, neuroticism, a personality disposition including anxiety, hostility, depression, insecurity, and inadequacy, has a more general and pervasive effect at all ages leading to increased complaints in all somatic systems (Costa & McCrae, 1980).

Somatic complaints and blood pressure

Several investigators (e.g. Stewart and Lond, 1953; Bulpitt *et al.*, 1977) have found that subjects who have been diagnosed as hypertensive report a variety of symptoms. Wood *et al.* (1978) found that hypertensives reported significantly more physical and psychological symptoms on the Cornell Medical Index (CMI) than normotensive controls. However, they found no association between number of somatic complaints and blood pressure among hypertensives. Costa *et al.* (1980) found a significant cross-sectional correlation between

the number of physical symptom reports on the CMI and blood pressure. This relationship disappeared, however, after accounting for the influence of age. Furthermore, using average blood pressures to predict somatic complaints from 4 to 12 years later, they failed to find any long-term influence of blood pressure on somatic complaints. Because little support has been found for an association between somatic complaints and blood pressure, somatic complaints among hypertensives is probably not attributable to their actual blood pressure levels.

Symptom reporting and hypertension awareness

Stewart and Lond (1953) provided the first evidence for the importance of hypertension awareness. They found that a majority of subjects who were aware of their diagnoses reported headache symptoms, while only a minority of subjects who were unaware of their diagnoses reported these symptoms. Kidson (1973) also examined the influences of hypertension awareness on symptom reporting by comparing hypertensive outpatients with randomly selected controls. He found that aware patients with elevated diastolic pressures reported more somatic complaints than their matched controls. Presuming that the controls were unaware of their hypertension, these results suggest that the differences in number of symptom reports were due to hypertension awareness.

Berglund *et al.* (1975) examined symptom reporting in a random sample of 50-year-old men drawn from a Swedish town. They found that untreated hypertensives who were unaware of their diagnoses did not report significantly more symptoms than normotensive controls, while treated hypertensives reported more symptoms than untreated hypertensives. These results provide evidence for the similarity in symptom reporting between unaware hypertensives and normotensives. In other words, differences in the number of somatic complaints reported by aware and unaware hypertensives are probably more attributable to medical treatment (e.g. receiving a diagnosis or prescription for treatment) than to objective medical status.

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Effects of hypertension diagnosis on self-rated health

Fewer studies have examined the impact of a history of hypertension diagnosis on global health ratings or on self-rated health. Receiving a diagnosis for a potentially life-threatening disease such as hypertension could produce psychological distress, increased somatic concern, and lower ratings of global health. For example, Bloom and Monterossa (1981) examined self-rated health in normotensives who had previously received hypertensive treatment and a control group of normotensives who had never received hypertensive diagnoses. They found that the normotensives who had previously been labeled with hypertensive diagnoses rated themselves as significantly less healthy than the controls.

Subjective ratings of health have been shown to predict mortality, even after controlling for objective health status. In fact, Mossey and Shapiro (1982) found that the risk of subsequent death was almost 3 times greater for elderly subjects who rated their health as poor than for those who rated their health as good. Among the elderly, Tissue (1972) found that self-ratings of health were more strongly related to objective health measures than to morale or general self-image. One interpretation of these results suggests that self-ratings of health may be less strongly related to emotional state and more strongly related to objective health measures than somatic complaint measures such as the CMI.

The present study

The purpose of the present study is to clarify previous conflicting findings regarding the effects of measured blood pressure, history of hypertension diagnosis, age, and neuroticism on number of somatic complaints and self-rated health in a non-health-care-seeking sample of adult men and women. Specifically, the goals of the study are (1) to determine the effects of being labeled as hypertensive on somatic complaints and self-rated health; (2) to determine whether there is an interaction between the personality dimension of neuroticism and awareness of hypertension on somatic complaints and self-rated health; and (3) to determine whether these relationships are similar in separate age groups.

METHODS

Subjects

Participants in the Baltimore Longitudinal Study of Aging (BLSA) are a community-dwelling, generally healthy group of volunteers who have agreed to return for medical and psychological testing at fixed intervals (Shock *et al.*, 1984). The sample has been recruited continuously since 1958, and the majority (96%) are white; work in, or are retired from, scientific, professional, or managerial positions (80%); graduated from high school (93%) or college (71%); and are married (88%). Although not representative of the general population in education or occupation, comparisons with a national probability sample (National Health and Nutrition Examination Survey Epidemiologic Followup Study) suggest that the BLSA sample does not differ markedly with regard to three personality dimensions of Neuroticism, Extraversion, and Openness to Experience (Costa *et al.*, 1986). Because studies of women were introduced in 1978, fewer women were participants when the data for the present study were collected. For the purposes of this paper, data were available on 251 women and 719 men; only first administration data are examined here. Subjects ranged in age from 17 to 95; men (mean = 50.0, SD = 17.9) were significantly younger than women (mean = 55.4, SD = 18.0).

Procedures

Every 2 years, BLSA participants reside at the Institute for 2½ days where they undergo a comprehensive examination which includes tests of their physiological, biochemical, and psychological functioning. Eight recumbent diastolic and systolic blood pressure readings are taken at ten minute intervals on two separate mornings by the resident nursing staff. Average systolic pressures ranged from 90 to 203 (mean = 127.2, SD = 17.7), and men and women were not significantly different. Average diastolic pressures ranged from 54 to 118 (mean = 79.2, SD = 9.5), and men (mean = 79.9, SD = 9.5) had significantly higher diastolic pressures than women (mean = 76.9, SD = 9.3). Subjects were classified as hypertensive if their mean systolic pressure was greater than or equal to 140 or if their diastolic pressure was greater than or equal to 90. According to these criteria, 24.1% of the sample (173 men, 60 women) were classified as hypertensive. The average difference between the hypertensive and normotensive groups was 13.2 mm Hg diastolic pressure and 31.9 mm Hg systolic pressure.

Subjects' histories of hypertension diagnosis was determined by positive responses to items on self-administered questionnaires concerning their medical history. Table 1 shows the distribution of hypertension diagnosis into age decade categories. 247 subjects (160

TABLE 1. DISTRIBUTION OF HYPERTENSION DIAGNOSIS BY AGE DECADES.

Age	N	% Total Sample	% Within Age Decade
<35	253	2.8	10.7
35-44	143	2.5	16.8
45-54	153	4.5	28.8
55-64	135	4.9	35.6
65-74	181	6.7	35.9
≥75	105	4.0	37.1

men, 87 women) had a history of hypertension diagnosis, which, as the table shows, is increasingly prevalent as a function of age. As expected, some of the subjects (31 men, 33 women) in the normotensive group also reported a history of treatment for hypertension.

Information about somatic complaints was collected by administering the Cornell Medical Index (CMI; Brodman *et al.*, 1949), a self-report symptom checklist with 195 items divided into 12 somatic sections (A–L) and 6 psychiatric sections (M–R). The sum of somatic sections yields a measure of total somatic complaints. In this study, cardiovascular symptoms were removed from the sum of the somatic sections to avoid potential confounds with the independent variables. The sum of the psychiatric section yields a measure of psychological complaints or neuroticism. This measure correlates with neuroticism scores derived from personality inventories ($r = 0.52$; see Costa and McCrae, 1980). In this study, subjects were classified as high in neuroticism if they had 2 or more positive responses to items in the CMI psychiatric section. According to this criterion, 48% of the sample (294 men, 169 women) were classified as high in neuroticism.

Information about self-related health status was collected by administering the Activities and Attitudes Questionnaire (AAQ; Caven *et al.*, 1949), an inventory containing 158 items regarding general health status, family relationships, social participation, and friendships. 907 subjects rated their health as either "very poor" (0.4%), "poor" (0.7%), "fair" (8.0%), "good" (49.0%) or "excellent" (41.9%). Subjects who rated their health as poor also reported more physical symptoms on the CMI ($r = 0.39$; $p < 0.001$).

RESULTS

The effects of measured blood pressure, neuroticism, and hypertension diagnosis on number of somatic complaints and self-rated health were examined by a three-way analysis of variance covarying sex and age. These results are summarized in Table 2. Consistent with prior research, no significant main effects were found for measured blood pressure on either measure of health perception. However, significant differences were found for the other two main effects. For number of somatic complaints, significant differences were found due to neuroticism ($F[1,951] = 108.0$; $p < 0.001$) and hypertension diagnosis ($F[2,951] = 4.0$; $p < 0.05$). A significant two-way interaction was also found between

TABLE 2. SUMMARY OF EFFECTS FROM THREE-WAY ANALYSES OF VARIANCE ON NUMBER OF SOMATIC COMPLAINTS AND SELF-RATED HEALTH BY MEASURED BLOOD PRESSURE, NEUROTICISM, AND HISTORY OF HYPERTENSION DIAGNOSIS

Effect	Variable	
	Number of Somatic Complaints (N=965)	Self-rated Health (N=907)
Blood Pressure (B)	ns	ns
Neuroticism (N)	p<.001	p<.01
Hypertension Diagnosis (H)	p<.05	p<.001
B × N	ns	ns
B × H	ns	ns
H × N	p<.01	ns
B × H × N	ns	p<.01

neuroticism and hypertension diagnosis ($F[2,951] = 6.1$; $p < 0.01$). For self-rated health, significant differences were found for neuroticism ($F[1,893] = 8.1$; $p < 0.01$) and hypertension diagnosis ($F[2,893] = 10.8$; $p < 0.001$). There were no significant two-way interactions on self-rated health; however, a significant effect was found due to the three-way interaction ($F[2,893] = 6.2$; $p < 0.01$).

Because measured blood pressure had no significant main effect on either health perception measure, it was eliminated in subsequent analyses. Age (under 65 *vs.* 65 and over) was used to classify subjects in combination with neuroticism and history of hypertension diagnosis in a three-way analysis of variance on number of somatic complaints and self-rated health covarying sex. Table 3 summarizes the results of this analysis for number of somatic complaints. Significant differences were found due to age ($F[1,961] = 7.25$; $p < 0.01$), neuroticism ($F[1,961] = 154.17$; $p < 0.001$), and history of hypertension diagnosis ($F[1,961] = 11.98$; $p < 0.01$). A significant interaction was also found between neuroticism and history of hypertension diagnosis ($F[1,961] = 9.47$; $p < 0.01$).

Figure 1 displays the mean numbers of somatic complaints in each group of the three-way classification. History of hypertension diagnosis was associated with about 10 somatic complaints in the low neuroticism group and about 19 somatic complaints in the high neuroticism group. With the exception of the effect of neuroticism, which accounted for approximately 12% of the variance, the magnitudes of the effects of the independent variables (ω^2, shown in Table 3) on number of somatic complaints were quite small.

The results for the three-way analysis of variance on self-rated health are summarized in Table 4. As in the previous table, all three main effects were significant, but there were no significant interactions. Significant differences were found due to age ($F[1,902] = 9.46$, $p < 0.01$), neuroticism ($F[1,902] = 20.67$; $p < 0.001$), and history of hypertension diagnosis ($F[1,902] = 23.27$; $p < 0.001$).

Figure 2 displays the means for self-rated health by the three-way classification. History

TABLE 3. SUMMARY OF EFFECTS AND EFFECT SIZES FROM THREE-WAY ANALYSIS OF VARIANCE ON NUMBER OF SOMATIC COMPLAINTS BY AGE, NEUROTICISM, AND HISTORY OF HYPERTENSION DIAGNOSIS.

Effect	F	ω^2
Age (A)	7.25**	.005
Neuroticism (N)	154.17***	.125
Hypertension Diagnosis (H)	11.98**	.009
A × N	1.81	
A × H	2.31	
N × H	9.47**	.007
A × N × H	0.55	

Note: $^{**}p < 0.01$; $^{***}p < 0.001$; $N = 970$; ω^2 assesses the size of the treatment effect which is interpretable as the proportion of total variance accounted for by an independent variable.

of hypertension diagnosis was associated with an average self-health rating between "excellent" and "good" in the low neuroticism group and an average self-rating around "good" to "fair" in the high neuroticism group. The magnitudes of the effects of the independent variables (ω^2, shown in Table 4) on self-rated health were only modest.

To determine whether these results depend on the particular cut-off values chosen for grouping subjects into blood pressure and neuroticism groups, these data were reanalyzed using different cutting scores for classifying subjects. The results were unchanged when

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TABLE 4. SUMMARY OF EFFECTS AND EFFECT SIZES FROM THREE-WAY ANALYSIS OF VARIANCE ON SELF-RATED HEALTH BY AGE, NEUROTICISM, AND HISTORY OF HYPERTENSION DIAGNOSIS.

Effect	F	ω^2
Age (A)	9.46**	.009
Neuroticism (N)	20.67***	.020
Hypertension Diagnosis (H)	23.27***	.023
A × N	1.73	
A × H	1.45	
N × H	0.24	
A × N × H	0.42	

Note: $^{**}p < 0.01$; $^{***}p < 0.001$; $N = 911$.

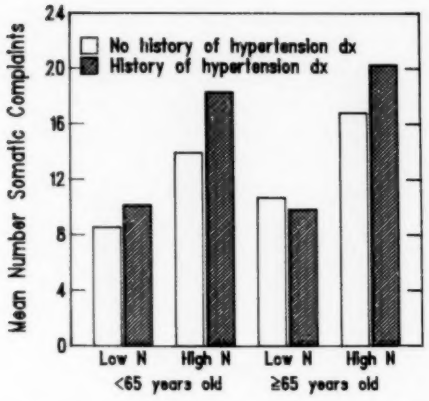

FIG. 1. Mean number of somatic complaints as a function of neuroticism, age, and history of hypertension diagnosis.

subjects with mean systolic pressures greater than or equal to 160 or mean diastolic pressures greater than 95 were classified as hypertensive. The results were also unchanged when subjects with 3 or more positive responses to items in the CMI psychiatric section were classified as high in neuroticism. Similarly, the results were unchanged when 4 or more positive responses was used as the cutting score for neuroticism groups.

DISCUSSION

The present results are broadly consistent with previous findings. Age, neuroticism, and history of hypertension diagnosis showed statistically significant effects on both measures of health perception. Measured blood pressure, however, was unrelated to either measure of health perception. Self-rated health status and somatic complaints were influenced by

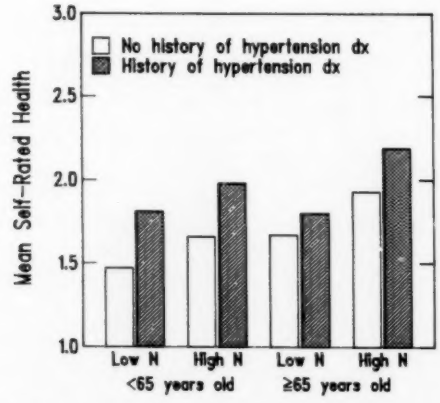

FIG. 2. Mean self-rated health as a function of neuroticism, age, and history of hypertension diagnosis. Note: Higher scores indicate poor health: 1 = excellent; 2 = good; 3 = fair.

psychological factors: neuroticism and awareness or labeling of hypertension as a consequence of its diagnosis; they were not influenced by actual blood pressure values. With the exception of the 12.5% of the total variance in somatic complaints accounted for by neuroticism, the magnitudes of the effects of the independent variables were uniformly quite small. It is especially important to note that age, though significantly related to both health perception measures, accounted for only 0.5% and 0.9% of the variance in somatic complaints and self-rated health, respectively. Similarly, history of hypertension diagnosis accounted for small amounts of variance, though it increased from 0.9% for somatic complaints to 2.3% of the variance in self-rated health. Neuroticism had a much greater effect on somatic complaints than on self-rated health despite the moderate correlation ($r = 0.39$) between somatic complaints from the CMI and the single-item global health rating.

One suggestive finding worth replication is the interaction of neuroticism with awareness of hypertension on number of somatic complaints. For well-adjusted subjects there is no consistent increase in somatic complaints given a history of hypertension diagnosis. But there is a consistent 25% increase in somatic complaints given a history of hypertension diagnosis for subjects prone to experience psychological distress. The negative impact of hypertension diagnosis on health perceptions and life-style reported in the literature is probably confined to individuals with enduring dispositions of neuroticism.

Self-related health did not show evidence of the interactive effect of history of hypertension diagnosis and neuroticism. Thus, depending on the way health perceptions are measured, different conclusions are possible regarding the moderating effects of neuroticism. It would appear that self-rated global health status is a complex, multidimensional construct, and that individuals have many different ways of assessing their own health. Age, neuroticism and history of hypertension diagnosis are clearly not its major determinants because almost 95% of the variance in self-rated health is unexplained.

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The situation is somewhat better for the CMI measure where almost 13% of the variance is explained by neuroticism; but including the other main effects reduces the error variance only by an additional 1%. At any rate, neuroticism and awareness of hypertension are more important predictors of somatic complaints than is age per se.

The very small effects of hypertension history or awareness might be surprising to clinicians who consider hypertension as a serious condition and understand its significance as a risk factor for stroke and other unwanted complications. The fact that individuals perceive hypertension as having little impact on their health might help to explain why many patients fail to comply with treatment. The small magnitude of effect on health perceptions can also be viewed positively. Reports that the discovery and diagnosis of hypertension have deleterious consequences for adult men and women's sense of health status find only limited support in the present study. The use of patient samples or worksite screening programs (where there are compensation and disability considerations) doubtless contributes to differences in findings. Participants in the BLSA are not self-selected for treatment seeking nor are there any incentives for adopting the sick-role. The present findings support physicians and other health care providers in diagnosing and treating elevated blood pressure without fear that psychological disability will necessarily ensue.

Diseases and medical conditions which are more symptomatic and immediately life-threatening (such as massive acute myocardial infarctions, and metastatic cancer) might well be expected to have greater effects on health perceptions. Asymptomatic conditions, however, provide few signals other than side-effects from treatment that would exert effects on self-definition of health or life-style. Important moderators such as the individual's coping mechanisms, social supports, prior health history need to be recognized as well.

Finally, further research needs to be conducted utilizing large representative samples and prospective designs that examine changes in health perceptions as a function of changes in hypertension status as well as other disease and medical conditions. Ideal studies would also measure the individual's subjective appraisal of the meaning of any health change or condition in order to better understand the determinants and significance of subjective health perceptions and aging.

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A LONGITUDINAL STUDY OF COGNITIVE PERFORMANCE IN NONINSULIN DEPENDENT (TYPE II) DIABETIC MEN

ELIZABETH A. ROBERTSON-TCHABO

Department of Human Development, University of Maryland, College Park, Maryland and Gerontology Research Center, National Institute on Aging, N.I.H., Baltimore, Maryland

DAVID ARENBERG, JORDAN D. TOBIN and JUDITH B. PLOTZ

Gerontology Research Center, National Institute on Aging, N.I.H., Baltimore, Maryland

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Abstract—Diabetic men (noninsulin dependent, Type II) in the Baltimore Longitudinal Study of Aging were compared with nondiabetics on two cognitive performance tests—the Benton Visual Retention Test, a measure of nonverbal memory, and the Vocabulary subtest of the Wechsler Adult Intelligence Scale. Cross-sectional comparisons of 52 diabetics with matched controls and with 610 controls (covaried for age and education) found no group differences and no support for an accelerated cognitive aging effect of diabetes. In addition, longitudinal comparisons over six years and over twelve years found no effect of diabetes on change in cognitive performance. Possible reasons for disagreements of cross-sectional findings in the literature are discussed. One possibility is that only specific aspects of cognitive performance are adversely affected by diabetes. It is also possible that lower socio-economic status (as indexed by education) may be a risk factor in the effect of diabetes on cognitive performance, perhaps by delaying diagnosis and treatment.

Key Words: longitudinal study, cognition, diabetes, noninsulin dependent diabetes, age differences, Benton Visual Retention Test, vocabulary

INTRODUCTION

THE PURPOSE of this paper is to compare age differences and age changes in cognitive performance between noninsulin dependent (Type II) diabetic men and normal, healthy controls. These men were volunteer participants in the Baltimore Longitudinal Study of Ag-

Correspondence to: David Arenberg, Gerontology Research Center, National Institute on Aging, N.I.H., Baltimore, MD 21224.
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ing (BSLA), established by Dr. Nathan Shock in 1958 and directed by him for many years (see Shock *et al.*, 1984).

Under Dr. Shock's leadership, the primary objective of the BLSA was the empirical characterization of aging in individuals over the entire adult life span. Unlike epidemiological studies whose chief purpose is to identify factors that increase the probability of the development of specific diseases, such as cardiovascular disease, the BLSA emphasizes normal, healthy aging. The BLSA has included a broad spectrum of both physiological (e.g., glucose metabolism) and psychological tests (e.g., nonverbal memory, verbal learning, vocabulary, problem solving, and reaction time). Although one goal was to rule out clinical disease as a source of observed age-related differences and changes, subjects with health problems were not excluded from the study. As a result, the progressive effects of such conditions as diabetes and cardiovascular disease on specific psychological processes can be described. Dr. Shock believed that the BLSA afforded a unique opportunity to examine the multidisciplinary inter-relationships among variables and conditions.

Glucose tolerance is known to decrease with advancing age (Andres, 1971; DeFronzo, 1981; Tobin, 1984). Interpretation of this decline in glucose tolerance has been controversial. Some have argued that the decline represents a true maturational effect, but others have viewed the decline as the emergence of diabetes mellitus. Partly in response to suggestions that the criteria for the diagnosis of diabetes mellitus be age-adjusted, in 1979 the National Diabetes Data Group published a revised classification of diabetes and other categories of glucose intolerance. Type II, noninsulin dependent diabetes mellitus is a condition, not secondary to other diseases or conditions, that frequently has minimal or no symptoms (e.g. polyuria, polydipsia) referable to the metabolic aberrations of diabetes. Type II diabetics are not dependent upon insulin for prevention of ketonuria and are not prone to ketosis. However, they may require insulin for correction of symptomatic or persistent fasting hyperglycemia if this condition cannot be corrected by diet or by use of oral agents. Type II diabetics may develop ketosis under special circumstances, such as severe stress precipitated by infection or trauma. Individuals with Type II diabetes may be asymptomatic for years or decades and show only slow progression of the disease. However, the typical chronic associations and complications of diabetes (macroangiopathy, microangiopathy, neuropathy, and cataracts) are found in both Type I and Type II diabetics. Although the onset of Type II diabetes is usually after age forty years, it also occurs in young persons who do not require insulin and are not ketotic. Type II diabetes has a genetic basis as evidenced by a frequent familial pattern of occurrence. Environmental factors superimposed on genetic susceptibility undoubtedly are involved in the onset of Type II diabetes. Obesity is probably an important factor in its pathogenesis, and Type II diabetics have been divided according to whether or not obesity is present. In Western societies 60–90% of all Type II diabetics are obese.

Recently, Perlmuter *et al.* (1984) compared the cognitive performance of patients with Type II diabetes mellitus selected from a diabetic clinic with a comparably aged, nondiabetic control group selected from other outpatient clinics. The results indicated that for two tasks (serial learning and backward digit span) the diabetics performed significantly more poorly than the controls. The authors pointed out that the cognitive impairment apparent on such a learning task may complicate adherence to a medical regimen. Moreover, they endorsed previous suggestions that the aging process, in general, and cognitive functioning, in particular, may be accelerated in diabetics.

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That was a cross-sectional study. Questions concerning the rate of change within an individual, however, can be addressed only by using a longitudinal design. The BLSA afforded an unique opportunity to address the question of differences between diabetic and nondiabetic men in the rate of change in cognitive performance. We selected Benton's (1963) Revised Visual Retention Test (Benton VRT) and the Vocabulary subtest of the Wechsler Adult Intelligence Scale (Wechsler, 1955) as indices of cognitive performance for two reasons. First, descriptive longitudinal data for the BLSA study group have been published previously (Arenberg, 1978). In general, the results indicated substantial age declines in memory-for-designs performance (Benton VRT) only for men aged seventy years or more, but only small (if any) age declines in vocabulary test performance. Second, these two tests of cognitive performance have been administered since 1960 and there are more times of measurement available for these instruments than for other cognitive measures.

Dr. Shock appreciated the potential of the BLSA to address research questions that require a multidisciplinary data base, and he encouraged researchers at GRC to pursue such questions. This study represents one such effort.

METHOD

Subjects

Fifty-two men were identified who had enrolled in the Baltimore Longitudinal Study of Aging (BLSA) before 1978 and who were, at the time of their first visit to the Gerontology Research Center, noninsulin dependent diabetics (using the criteria of the National Diabetes Data Group, 1979). They ranged in age from 33.0 to 86.7 years at the time of their first measure of these cognitive tests (mean age = 62.2 years). As a group, these men had a high level of formal education—65% of the subjects held a bachelor's or higher degree.

The group of diabetic subjects was divided into two subgroups based on additional indices of health status at the time of their first cognitive performance measure. The charts were reviewed for medications for other chronic diseases (including diuretics, antihypertensives, amphetamines, digitalis, coronary and other vasodilators, cardiac drugs, major tranquilizers, tricyclic antidepressants, psychostimulants, ergot alkaloids, alpha-adrenergic blockers, L-Dopa, monamine oxidase inhibitors, and specified xanthine derivatives) and for other diseases or markers (creatinine clearance, coronary heart disease, definite cerebrovascular disease, anemia, and cancers with systemic effects). Using these criteria, 37 of the 52 diabetic subjects were considered to have no clinical condition (e.g. arterial hypertension) in addition to diabetes mellitus that would be expected to have a direct effect on cognitive performance. Test scores were available for a second time of measurement at least six years later for 25 of these 37 diabetics. Applying the screening criteria described above, 16 of the 25 men continued to be free of other clinical conditions or medications. Nine of the men at the second time of measurement of cognitive performance had developed other diseases that also might have affected cognitive performance.

In addition, two nondiabetic control groups were identified: matched-pair controls and all controls. Every control subject met the health criteria described previously at the BLSA visit for which the first cognitive performance measures were obtained. One of the reasons that we decided to use a matched pairs comparison was that the diabetics were over represented in the lower educational levels in the BLSA. The matched-pair control

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subjects were case-controlled for age, level of formal education, and time of entry into the study. In comparisons of diabetic subjects and all controls, age and educational level were controlled statistically.

Procedure

All subjects were given Form C, Administration A of the Benton VRT (Benton, 1963) at the first time of the measurement (usually on their first 2½ day visit to the BLSA). At the second time of measurement, six (or more) years later, all subjects were given Form E; at the third time of measurement, 12 (or more) years after the first, all subjects were given Form D; and at the fourth time of measurement, 18 (or more) years after the first, all subjects were given Form C.

Each form of the Benton VRT consists of ten designs. The first two designs each consist of one major geometric figure. Each of the remaining eight complex designs consists of two major figures and a smaller, peripheral figure. Using Administration A, each design is displayed for ten seconds and removed. The task is to draw the design from memory and is self-paced; that is, there is no time constraint for completion of the reproduction. The test was scored according to the test manual. Each design was scored independently by two psychologists, and the infrequent disagreements were resolved by discussion or by a third psychologist. The dependent variable was the total number of errors committed in reproducing the ten designs in a form.

Whenever a form of the Benton VRT was administered, a subject also received the vocabulary subtest of the Wechsler Adult Intelligence Scale (Wechsler, 1955). The vocabulary subtest was scored independently by two psychologists according to the test manual. The dependent variable was the raw score for the vocabulary subtest (the maximum possible score being 80).

The data were analyzed in several different ways. All cross-sectional comparisons were based on first measures. The diabetics were compared with matched-pair controls (matched for age and education) and with all controls; in the latter analyses, age and education were covariates.

The longitudinal analyses were of two kinds: (1) comparisons of group changes between two times of measurement; and (2) for subjects with three or more times of measurement, comparisons of groups using individual regressions (slopes) as the dependent measure.

All analyses were carried out both for all diabetics and for the subgroup of diabetics who were screened for several other health variables.

RESULTS

Cross-sectional

Means and sample sizes for all diabetics, their matched-pair controls, the subgroup of screened diabetics, their matched-pair controls, and all controls are shown in Table 1 for both cognitive variables and for age. The proportions of subjects with at least a bachelor's degree were (a) 0.65 for all diabetics (and their matched controls), (b) 0.67 for the screened subgroup of diabetics (and their matched controls), and (c) 0.78 for all controls.

For all diabetics, means for vocabulary raw score and Benton VRT errors were quite similar to those of their matched controls. The results for the screened subgroup were

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TABLE 1. CROSS-SECTIONAL MEANS

	N	All Diabetics	Matched Controls	N	Screened Diabetics	Matched Controls	N	All Controls
Age	52	62.2	62.0	37	59.2	58.9	610	48.3
WAIS Vocabulary	52	64.5	64.8	37	64.2	65.6	606	65.0
Benton VRT	51	5.25	5.71	36	4.89	5.25	610	3.77

similar to those for the entire group. Furthermore, both the full group and the screened subgroup of diabetics were similar to the unmatched control group on vocabulary. Although both diabetic groups were higher than the unmatched control group on Benton VRT errors, these differences were virtually eliminated after adjustment for age and education in the analyses of covariance. Both diabetic groups were substantially higher in age and somewhat lower in education than the unmatched controls.

A corollary of the hypothesis that diabetes leads to accelerated aging is that the slope of the regression of first measures of cognitive performance on age should be steeper for diabetics than for controls. Those slopes (increase in errors per year) for Benton VRT errors were (a) 0.115 for all diabetics, (b) 0.127 for their matched controls, (c) 0.090 for the screened diabetics, (d) 0.101 for their matched controls, and (e) 0.094 for the unmatched controls. These results are not consistent with the hypothesis of accelerated aging.

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Longitudinal

The longitudinal data were analyzed in three ways: (a) change from Time 1 to Time 2, (b) change from Time 1 to Time 3, and (c) individual slopes for those participants with three or more measures.

Time 1 to Time 2. Means for the five groups at Time 1 and Time 2 are shown in Table 2. On the Benton VRT, mean errors increased 1.24 for the total diabetic group and 0.31 for their matched controls. That difference was not statistically significant, $F(1, 28) = 1.24$. For the health-screened subgroup, mean errors increased 1.36 and decreased 0.20 for their matched controls. When this group was divided further into those who continued to meet the health criteria and those who did not, the difference was clearly attributable to the latter group and their controls. Mean errors increased 0.88 for the 16 diabetics who maintained their health status and 0.56 for their matched controls. For the 9 diabetics who no

TABLE 2. LONGITUDINAL MEANS: TIME 1 AND TIME 2

	All Diabetics			Matched Controls		Screened Diabetics			Matched Controls		All Controls		
	N	1	2	1	2	N	1	2	1	2	N	1	2
WAIS Vocabulary	31	64.6	63.9	65.7	65.4	25	64.2	64.0	66.6	66.1	415	65.1	65.1
Benton VRT	30	4.03	5.50	4.40	4.67	25	4.24	5.60	4.44	4.24	412	3.47	4.07

TABLE 3. LONGITUDINAL MEANS: TIME 1 AND TIME 3.

	All Diabetics			Matched Controls		Screened Diabetics			Matched Controls		All Controls		
	N	1	3	1	3	N	1	3	1	3	N	1	3
WAIS Vocabulary	22	64.9	63.6	66.7	63.9	20	65.0	64.2	67.4	64.5	316	65.0	64.8
Benton VRT	22	3.95	4.59	4.18	5.82	20	3.95	4.65	4.15	5.80	316	3.23	3.90

longer met the health criteria at Time 2, mean errors increased 2.22 and decreased 1.56 for their matched controls. This three-way interaction (involving time of measurement, diabetic subgroup, and matched controls) was statistically significant, $F(1, 23) = 4.58$, $p < 0.05$. Only the subgroup of diabetics who no longer met the health criteria at the second time of measurement were significantly different from their matched controls. Analyses of covariance comparing the diabetic groups with the unmatched control group were all negative, Fs < 1.

No difference was found in any of the comparisons of diabetics with matched or unmatched controls on the vocabulary measure.

Time 1 to Time 3. All of the analyses comparing diabetics and controls on change in performance between Time 1 and Time 2 (at least six years) were carried out for those participants with measures at least twelve years after the first. Means for Time 1 and Time 3 are shown in Table 3. None of the differences between groups was statistically significant for either the Benton VRT or the vocabulary score.

Individual slopes. Another measure of an individual's change in performance is the slope of his measures over time. Individual slopes (change per year) were calculated for all participants with at least three times of measurement. The means are shown in Table 4 for the five groups (all diabetics, their matched controls, the screened subgroup of diabetics, their matched controls, and all controls). None of the comparisons between diabetics and their matched controls was statistically significant. When the diabetic groups were compared with all controls, the diabetics declined more; but when these data were covaried for age and education, the differences virtually vanished.

It is possible that the failure to detect differences in cognitive performance between the diabetic and control groups in the BLSA was due to the severity of the disease. Not all of the diabetics in this study were under treatment. Therefore, the cross-sectional, ($N = 24$), six-year longitudinal ($N = 13$), and slope analyses ($N = 8$) were repeated for only those diabetics who were being treated for the disease at the time of their first Benton VRT and vocabulary measures. None of the analyses indicated an effect of the disease.

TABLE 4. LONGITUDINAL MEANS: INDIVIDUAL SLOPES.

	Mean Slope			Mean Slope			Mean Slope	
		All	Matched		Screened	Matched		All
	N	Diabetics	Controls	N	Diabetics	Controls	N	Controls
WAIS Vocabulary	22	-0.11	-0.15	19	-0.06	-0.16	304	0.00
Benton VRT	22	0.08	0.12	19	0.07	0.11	302	0.04

DISCUSSION

In general, our results do *not* support the position that cognitive performance is poorer in men with noninsulin dependent (Type II) diabetes compared with nondiabetic control subjects matched for age and educational level. Moreover, there was no evidence in our sample that the rate of change was greater for the diabetic men compared with matched pairs or with all controls. Longitudinal analyses are subject to survivor bias; that is, those men who live and return for repeated measures generally may be better performers at the first time of measurement. However, we also did not find differences between diabetic and nondiabetic men in our cross-sectional analysis of first time measures. Other cross-sectional studies have been reported with inconsistent findings. Mattlar *et al.* (1985) did not find differences between diabetic and nondiabetic men and women for several cognitive tasks including the Benton VRT and the vocabulary subtest of the WAIS. However, Meuter *et al.* (1980) reported that in a cross-sectional study of 35 case-controlled diabetics differences were found for a speed of reaction factor and for a memory-concentration factor, but differences were not found for two other factors. The Benton VRT was one of the tests included in their one hour psychological test battery. Diabetes apparently does not accelerate the aging of cognitive processes universally. At most, diabetes affects cognitive performance selectively.

One possible reason for the conflicting findings is that researchers have employed different cognitive tasks. Perlmuter *et al.* (1984) found performance differences between diabetics and nondiabetics for serial learning and for backward digit span but not for several other tasks. Meuter *et al.*(1980) found differences between diabetics and nondiabetics for a memory-concentration factor. Although the factor analysis of the psychological tests was not presented in the paper, the psychological tests included the Digit Span subtest of the WAIS, paired-associate learning, and a nonstandard administration of the Benton VRT. Assuming that these were among the tasks that defined the memory-concentration factor, it is possible to speculate about which components of cognitive performance are affected by diabetes. Both serial and paired-associate learning typically are paced tasks. Pacing requires a subject to respond within a fixed time limit and is a condition that has been demonstrated to affect disproportionately the older subjects (e.g. Arenberg, 1965). It may be that diabetics are particularly penalized when a task places a premium on speed of response. Moreover, the anticipation method requires a subject to alternate attention between registration and retrieval of information. In other words, the timing of sequencing of task elements is important to maximize performance. The digit span task also requires a subject to hold information in storage and at the same time to add new digits to the list of to-be-remembered items. In addition, backward digit span requires a subject to manipulate stored information. It is important to note that the dialogue between a diabetic and a health care provider is a paced task. The diabetic must process important information at the rate at which a health care provider speaks (see McNeal *et al.*, 1984).

Whether differences in cognitive performance between diabetics and nondiabetics are detected in a particular study also may be related to characteristics of the specific sample (sex, age, and educational level). Perlmuter *et al.* (1984) recruited 140 Type II diabetics and 38 controls from the outpatient population at the Massachusetts General Hospital. These men and women were aged 55 to 74 years. The average educational level for the groups was a high school graduate (means were 11.7 years for the diabetics and 12.4 years for the controls). Meuter *et al.* (1980) studied 35 diabetic men and women and control pairs

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matched for sex, age, and educational level. The diabetics were patients at the Diabetes Research Institute and the controls were employees of industrial companies or friends and relatives of the Institute staff. There were three levels of education based on stages of school graduation. Twenty-nine of the 35 pairs were in the low and middle education groups. Mattlar *et al.* (1985) selected 33 diabetic men and women and paired controls matched for age and sex from a large epidemiological and clinical study in the district of the Turku (Finland) University Central Hospital. The subjects were not matched for educational level, but 29 of the diabetics and 32 of the controls had completed at least elementary schooling. In the BLSA sample, the 52 diabetic subjects, all men aged 33.0 to 86.7 years, were not recruited from a hospital setting. In general, the men had higher levels of formal education than the subjects in the studies mentioned above, 65% of the men having at least a bachelor's degree. However, it should be noted that although only one of our subjects had not earned a high school diploma, 17 other subjects had less than a bachelor's degree. The other two studies which found differences in cognitive performance between diabetics and controls recruited subjects from diabetic treatment centers (Perlmuter *et al.*, 1984 and Meuter *et al.*, 1980). It may be that these patients are not representative of all diabetics but rather include individuals with more severe diabetes. Moreover, the level of formal education of the diabetics in the studies finding differences between diabetics and controls in cognitive performance was lower than that of the BLSA men. Formal educational level is correlated with level of performance on cognitive tasks and was an important predictor of performance for our BLSA sample. To the extent that educational level is correlated with access to and utilization of health care services, the diabetics in the Meuter and Perlmuter studies may have had unrecognized and untreated diabetes for longer periods of time than the BLSA diabetics. It seems likely that prolonged hyperglycemia, a major factor contributing to the neurologic and vascular complications of diabetes, may be related to the cognitive impairment observed by Meuter *et al.* (1980) and Perlmuter *et al.* (1984).

In conclusion, not all cross-sectional studies of cognitive performance found an effect of diabetes. Moreover, in this, the only longitudinal study of diabetes and cognitive performance, no evidence was found for an increased decrement in Benton VRT or vocabulary performance for diabetics. None of our results is consistent with a model of diabetes as accelerated aging. Despite these negative findings, it is important to recognize that without Dr. Shock's comprehensive planning it would not have been possible to examine these cognitive changes and to relate them to diabetes.

Acknowledgment—Since 1960, many psychologists have participated in administering and scoring the tests included in these analyses, most recently, Judy Friz, Barbara Hiscock, and Susan Robinson. Under the direction of Dr. Reubin Andres, many physicians and physiologists have participated in the evaluation of the volunteers of the BLSA. The authors gratefully acknowledge their contributions and those of the BLSA participants.

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FORTY YEARS OF EXPERIMENTAL PSYCHOLOGY IN RELATION TO AGE: RETROSPECT AND PROSPECT

A.T. WELFORD

Hadsley House, Lefebvre Street, St. Peter Port, Guernsey, Channel Islands

Abstract—Experimental psychological research on performance in relation to age is outlined to show how aims, methods and interests have developed between the pioneering days of the 1940s and the present time. More detailed consideration is then given to the actual and potential applications to studies of aging of the quantitative methods of Information and Signal Detection Theories, and to the importance of taking account not only of correct performance but of the frequency and nature of errors if age changes are to be fully understood. Research in relation to age on continued performance is discussed, including short-term serial effects and the longer terms effects of fatigue, learning and practice, and the need for further research in these areas is emphasised. Guiding principles for future psychological research on aging are briefly urged.

Key Words: aging, capacity, compensatory relationships, fatigue, industrial performance, information theory, learning and memory, longitudinal studies, serial performance, signal detection theory

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IT IS indeed a privilege to pay honour to Nathan Shock who has for many years been the world doyen of gerontology. I personally owe him a special debt. He was visiting Europe in 1950 and came to see our team in Cambridge. We were not there at the time but were at London Airport studying fatigue among aircrews in relation to age. Shock, however, came down to the Airport and was an inspiration to us who had for the past three and a half years been struggling to map out what was, at the time, the largely uncharted area of research on performance in relation to age. Later that year I visited him in Baltimore and he took me to the meeting at Pennsylvania State College of the American Psychological Association where he was promoting the study of gerontology to that body. We became, and have remained, firm friends.

EARLY DAYS AND IDEAS

When Shock came in 1950, our team, like that of W.R. Miles in the 1920s and 1930s, was studying human performance with a view to finding out what older people could best do in industry. The research combined laboratory experiments and industrial field work, each serving to guide and check the other. It was essentially a fact-finding exercise without attempts to prove or disprove particular theories. It was, however, carried out within the

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framework of the research on skills that had been going on for the past decade at the Cambridge University Psychological Laboratory under the late Sir Frederic Bartlett.

The skills approach implied that, although the studies were of human performance, they were conceived within a framework of psychology as a biological science ultimately rooted in physiology. More immediately important, the approach attempted to analyse and measure the components of complex performance without isolating them from their context. In practice this involved, first, fractionating the time taken by a performance and studying how each fraction changed with age and how the fractions interacted with each other and second, taking careful note of the frequencies and types of error made. The research was laborious, involving long hours of work such as measuring distances between "blips" on paper which had been drawn over a revolving drum. We reckoned that if we tested subjects for a week, we had to spend the next six months processing the results. The labour was, however, worth while. While the results were being read off, many ideas about what they might mean had time to arise, and anomalies which would have been masked by less detailed scrutiny stood out as needing explanation. In short, the reward was a greater insight than could have been attained by a single overall score for time taken or errors made. Two examples deserve mention.

In one, the subject had to plot, with a pointer on a grid, the position of a target on another grid. He made trials until he found the correct position, whereupon the target moved until he pressed a key. It then stopped and the cycle started again with the target in a new position. Subjects aged 45–82 made fewer successful plots in half an hour than did those aged 18–29, but they made fewer errors. They also took longer to stop the target once it had started to move, seeming to wait until it was in an "easy" position near the edge of the grid before stopping it. The older subjects appeared to be using a different *method* of performance, *compensating* for lower speed by increased accuracy, and spending extra time over one part of the task in order to make another part easier (Welford, 1958, pp. 65–69). Similar kinds of compensation were observed in the performance of older factory operatives.

A second example is provided by a tracking task in which the subject had to move a lever to keep a pointer aligned with a target which moved irregularly from side to side at rates from 38 through 67, 100 and 128 to 150 times per minute. Older and younger subjects all made the correct number of movements. Time on target is not a meaningful score at these speeds: more significant is the extent of responding movement. Among the younger subjects this rose to the extent required by the target movements as far as the third speed, but rose no further at the fourth and fifth. The older subjects showed the same pattern except that there was no increase of movement after the second speed. The results point to an important concept of *capacity* (Welford, 1978): performance is adequate until the demands of the task load the subject's capacity fully. Thereafter, performance fails to meet demand. The older subjects' capacities appeared to be less than those of the younger (Welford, 1958, pp. 86–89). The concept becomes more important when it is recognised that in sensory-motor performance, several capacities are involved but in any given situation it is usually only one of these that limits performance. Thus, in choice reaction tasks where movements are simple – pressing a key or moving a light lever – reaction times have been found to rise with age while movement times remain virtually unchanged (see Welford, 1958 pp. 76–82), whereas with simple reactions and more forceful movements, the movement times rise more than the reaction times (Pierson and Montoye, 1958; Onishi, 1966). Counterparts to these relationships have to be found in industry where, although people

tend to move from very strenuous work as they grow older (Powell, 1973), work involving moderately heavy muscular effort has been better supported by older operatives than work done under pressure for speed (Welford, 1958 pp. 113–128; 1966a). The consequences of such nonlinear relationships between demand and performance are, first, that we cannot expect any uniform and universal slowing or other change of performance with age; and second, that linear correlation methods are often inappropriate for studies of aging: instead, the need is to ascertain limiting levels and functional relationships.

FROM PAST TO PRESENT

With the rapid expansion of research on aging since the 1950s has come a change of emphasis. Links with physiology have strengthened in line with developments in that subject (Welford and Birren, 1965; Poon, 1980 Sec. 2–4). Industrial studies reached a peak in the late 1950s with much innovative research on both performance and training (Belbin, 1958; Belbin and Downs, 1966; Murrell *et al.*, 1957; Murrell and Griew, 1958; Welford, 1966a, 1976). They then declined although an important series of monographs was published in the 1960s by the Organisation for Economic Co-operation and Development (1965; Griew, 1964; Belbin, 1965, 1969). The immediate reason for the decline, at least in Britain, was revised population projections predicting that the labor shortage in the 1950s would be replaced by a substantial surplus of labor in the 1970s, so that earlier rather than later retirement would need to be encouraged to reduce unemployment. To one working at that time, however, the decline appeared to be part of a growing general aversion to applied research. The aversion seemed to have two causes. One was a post-war revulsion against anything to do with military activities, especially against classified research which was regarded as a negation of academic freedom and as precluding the publications on which an academic reputation could be built. The other, perhaps more basic human reason, was that field work is usually harder and less elegant than that in the laboratory, and requires social skills and qualities of personality that many laboratory workers find uncongenial.

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Another facet of the aversion to applied research appeared as an increased emphasis on theory. Instead of letting theory emerge inductively from facts, as the earlier workers had done, psychological departments, journal editors and higher degree examiners came to insist that research should be undertaken to test hypotheses deduced from pre-formed theories. Regarding laboratory experiments, the result was to reverse the practices of the earlier years in favour of studying isolated functions such as sensory thresholds or memory spans, and overall rather than fractionated performances, and to regard errors as a nuisance to be minimised or neglected rather than as a prime source of important data. Two further developments were, first, the widespread use of psychometric tests and questionnaires to compare age groups, and second, the initiation of several longitudinal studies (see Schaie, 1983). Outstanding among these for its breadth and thoroughness was—and is—the Baltimore Longitudinal Study of Aging (Shock *et al.*, 1984).

These developments have had both advantages and disadvantages. The laboratory experiments have become less laborious and have lent themselves to elegant statistical analyses. These have sometimes tended, however, to obscure rather than clarify data. Also, the leading ideas are often not original but derived from theories in the general field of psychology many of which are of questionable profundity, and the lack of detailed work on results has meant that they are viewed only as confirming or denying the theory

being tested—consideration is seldom given to whether the results might equally well bear on other theories.

Many questionnaires conducted in the name of psychology have been designed simply to obtain opinions or factual data, useful for particular purposes but of little general significance. A number of well researched psychometric tests with high standards of reliability and validity have provided valuable data on differences of cognitive ability (e.g. Wechsler, 1958) and on the stability of aspects of personality with age (Costa *et al.*, 1983). They have the difficulty, however, that they seldom throw much, if any, light on fundamental mechanisms of performance. For example, what essential mechanisms are involved in reproducing from memory a string of digits backwards—a task showing substantial decline associated with age—that are not involved in the task of reproducing the same string forwards, in which little decline occurs? Again, there is, as yet, no generally agreed definition, other than statistical, of intelligence, and attempts to link intelligence test scores to biological or other performance variables have been controversial (see Costa and Shock, 1980; Jensen and Vernon, 1986). Some attempts to link personality traits to biological variables have, however, been successful (see Welford, 1966b) and seem worth following up in relation to age.

Longitudinal studies have an obvious importance for physical and clinical variables, and enable age effects in these to be separated from cohort effects, but for psychological research they have the serious shortcoming that problem solving, performance at intelligence tests and even relatively simple functions such as reaction times, are profoundly affected by practice. Since, in longitudinal studies, performance must be repeated at different ages, practice effects are inevitably confounded with age and to a greater or lesser extent offset age effects, so that declines are underestimated. It might, at first sight, be expected that practice effects resulting from a first testing would have disappeared by the time of a second test made several years later. It is true that memory of items such as words learnt by rote usually declines rapidly, but motor skills such as riding a bicycle or using hand tools, once acquired, commonly remain little impaired over many years even though not practised during the interval. It seems likely that the same is true of what one may call *cognitive skills* or *insights* into ways of tackling problems. Insights of this kind are probably the explanation of the very substantial gains in intelligence test scores that have been found when a test is repeated at weekly intervals (Heim and Wallace, 1949). Despite the fact that no knowledge of results was given after testing, the gain of score from the first time of testing to the second substantially exceeded any decline likely to have been associated with age over a ten-year or even longer period. Generally speaking, when cross-sectional and longitudinal differences with age are similar, as for instance in the case of vigilance (Quilter *et al.*, 1983), there is strong evidence for change associated with age. When, as with intelligence tests (see Schaie, 1983), longitudinal differences are less than cross-sectional, practice effects must be considered as a possible explanation. Further studies of practice effects are needed, especially to distinguish between those which lead to the firmer registration of particular items and those which result in more generalised insights. It seems possible that older people tend to be slower at acquiring the latter, but that when they have done so they have little difficulty in using them. Slower acquision would impair performance the first time a task was met—as in a cross-sectional study—but might have little effect on later performances in a longitudinal study.

All in all, it seems fair to claim that in the great expansion of research on aging since the

1950s, the better psychological studies have maintained the tradition of straightforwardly seeking to understand the nature and causes of age differences in performance. Some of the rest have been less ambitious in aim, less efficient and have tended to drift with the general run of psychological fashion.

ATTEMPTS TO QUANTIFY

However, one theoretical concept from general psychology is emerging as important for explaining age changes of performance, and seems to act as a unifying concept for a wide range of findings. It is that performance results from decisions made on the basis of data which are confused to a greater or lesser extent by momentary random disturbances or "noise" either in the incoming signals or in the sensory mechanisms and central nervous system of the subject. The accuracy of the decisions depends on the signal-to-noise ratio attained when they are triggered. The model was first applied to aging studies by Crossman and Szafran (1956) and Gregory (1959, 1974). Its importance is that sensory impairments and changes within the central nervous system make it likely that signal-to-noise ratio will decline with age (see e.g. Welford, 1965a, 1981, 1984). The concept of signal-to-noise ratio is the basis of two closely related bodies of theorising – Information Theory (Shannon and Weaver, 1949) and Signal Detection Theory (Tanner and Swets, 1954; Green and Swets, 1966). Both were formulated by people who, like Shock, had been trained as engineers and neither is an essentially psychological theory. Rather, they are mathematical statements which apply widely in physical as well as biological science. They apply to psychology as overall measurements of performance without attempting to describe the detailed micro-behaviour which these embrace and which also needs to be studied if performance is to be understood fully. They have the important quality, however, of describing both task and performance in the same terms, which transcend particular tasks. They thus are attempts at true quantification in psychology.

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Information Theory, concerned to quantify the resolution of uncertainty, rose to prominence in psychology when it was shown by Hick (1952) that choice reaction time rose linearly with the amount of information transmitted from signal to response. The subsequent close and critical scrutiny to which this striking result led, has reduced the neatness and simplicity of the original picture, but its broad outlines remain clear. The relationship between choice reaction time and degree of choice has been expressed as depending on signal-to-noise ratio, rate of transmission of information from signal to response, and criterion level for responding (Smith, 1980). Different studies have indicated that age differences occur in each of these variables, although there is still work to be done to establish the exact conditions in which each applies (Welford, 1977a, 1984).

Similar in concept to what has come to be named as Hick's Law, is Fitt's Law that the time taken to make movements aimed at a target is proportional to the information extracted, as measured by the accuracy of aim in relation to the extent of the movement. The most extensive study of age differences in this, done in Shock's laboratory, showed that older and younger subjects differed little in information transmission rate but, more subtly, that from the 20s to the 40s there was an average gain of speed and loss of accuracy, while from the 50s onwards these tendencies were reversed. Overall, age differences appeared to be due partly to accuracy of hitting the targets and partly to distance moved, but rather more to the former than to the latter (Welford *et al.*, 1969; Welford, 1977a).

A corollary of Hick's and Fitt's Laws is that information can be gathered and accumulated over time. Older people have been found to do this when they are allowed time, and thus improve their signal-to-noise ratio (Vickers *et al.*, 1972). This is probably the reason why older people's performance has been found to suffer little from random disturbances to signals which would impair signal-to-noise ratio, if the time they take is not limited (Salthouse and Lichty, 1985). Also, the inverse relationship between time and accuracy emphasises the fact that Information Theory requires that errors are taken into account. For example, if the slope of choice reaction time against information is, when all responses are correct, say 150 msec. per bit and the intercept, say, 130 msec, a 2-choice reaction time would be 150 + 130 = 280 msec. With 2% of errors this would fall to 132 + 130 = 268 msec. and with 5% to 107 + 130 = 237 msec (Welford, 1980a, p. 76). These differences are of a magnitude comparable with those sometimes found between age groups and clearly illustrate the need to consider whether slowness of older subjects is at least partly due to increased accuracy.

Information Theory has been applied to other areas of performance such as perception, language and learning (e.g. Attneave, 1954; Davis *et al.*, 1961; Garner, 1962; Edwards, 1964) but not yet in relation to age. It has also been suggested as a means of measuring the work load of organisers and managers in terms of the number and frequencies of the sources of information and responding actions with which they are concerned (Leuba, 1967).

Signal Detection Theory has greatly clarified the requirements for reliable measurement of sensory thresholds, and has shown that many previous experimental and clinical determinations of these are seriously deficient. Measurement is essentially of *discrimination* in terms of signal-to-noise ratios and criterion levels of response. Poor discrimination by older people has been shown to be due in part to lower signal-to-noise ratios, but partly also to criteria that are too high (Craik, 1969; Rees and Botwinick, 1971; Potash and Jones, 1977; Watson *et al.*, 1979; Danziger and Botwinick, 1980; Hutman and Sekular, 1980). The distinction between the two sources of poor performance is important because, while the former can be overcome to a limited extent by stronger sensory stimulation such as improved lighting of visual tasks, the latter can be largely eliminated by suitable training.

Signal Detection Theory has been applied to several studies of recognition in relation to age (Craik, 1971; Gordon and Clark, 1974; Smith, 1975; Harkins *et al.*, 1979; Wallach *et al.*, 1980; Hess, 1984). Application has also been considered to recall, stress and motivation in relation to age (Welford, 1980b, 1981, 1985) but its potentialities in these areas are still largely unexplored, mainly because it is seldom easy to obtain the necessary data on which analysis can be based. For example, in studies of memory, it is necessary to take account not only of correct responses and errors, but of the frequencies of the latter in relation to the number of possible sources of error. In recognition this is easy because the number of distractors is known, but in recall this is not usually so. Methods of overcoming this difficulty are only just beginning to emerge (Welford, 1986).

Once again the importance of taking proper account of errors is emphasized. Suppose that in a recognition test the items previously presented are mixed with twice the number of distractors, and that 60% of the previously presented items are correctly recognised while 4% of the distractors are falsely "recognised": d' works out at 2.00. To obtain the same value of d' with 8% of errors would require the correct recognition of 72.5% of the previously presented items. Again the difference is comparable with some of those associated with age.

CONTINUED PERFORMANCE

Performance, whether in everyday life or in the laboratory, seldom consists of a single run through from input to output, but of a series of runs the outcome of each of which tends to influence those that follow. The serial effects in continued performance have long attracted attention in general psychology. Evidence in relation to age can be conveniently discussed under four headings, according to the time scale involved.

Immediate serial effects

Several experiments have found that reaction times within a series differ according to whether the signals and responses are the same as, or different from, those immediately preceding. These "repetition" and "alternation" effects also differ according to the time intervals between signals, the relations between signals and responses, and degree of choice in ways that are, as yet, not fully understood. However, Kirby (1980) on the basis of a thorough survey of the evidence, concludes that there must be some facilitating after-effect of a signal and response which enables a similar reaction to be made more quickly during the following second or so. It seemed reasonable to suppose that, since neural after-effects tend to last longer in older people, the facilitatory effect would be greater for them (Welford, 1965a). However, a series of direct tests of this hypothesis produced the diametrically opposite result – the older subjects' reactions to the repeated signals were, on average slower than to the different ones, and the differences were in almost all cases greater than those for younger subjects (Welford, 1977b). The results resembled those previously found with intervals between trials too long for neural after-effects to have been operating (Waugh *et al.*, 1973) Even when repetition effects have been found, they have tended to be less for older than for younger subjects (Jordan and Rabbitt, 1977).

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Repetition and alternation effects have been shown to be affected not only by immediately preceding reactions but by those 2, 3, and 4 steps back (Kirby, 1980). Age differences in these more remote effects are not known, but evidence from other tasks suggests that they are likely to be less for older subjects than for younger. For example, a serial reaction task which simulated the monitoring of an industrial plant, required subjects, for efficient performance, to take account of several previous signals. Older subjects were found to take account of fewer of these, seemingly because of a shorter span of "running" memory (Maule and Sanford, 1980), for which there is other evidence especially from a detailed study by Poon and Fozard (1980).

Extended serial effects

One of the most brilliant pieces of research on reaction times in relation to age is that of Rabbitt (1981) who found that serial choice reaction times gradually became shorter over a succession until an error was made, whereupon the next reaction was much longer. Over the next few reactions the subject again speeded up until another error was made, and so on. The slowing after an error was greater for older subjects than for younger. Rabbitt suggested that subjects "track" their reaction times to secure an optimum balance between speed and accuracy, going as fast as possible without making many errors – some errors must be made if optimum speed is to be gauged. He noted that older subjects were as able as younger to detect their errors, that their error reaction times were no longer than those of younger subjects although their correct reaction times were so, and that their reaction

times were more variable. He concluded that this variability meant that they had to maintain a larger "margin of safety" if they were to avoid errors, and that this was part of the reason for their generally longer correct reaction times. The increased variability is in line with the idea that signal-to-noise ratio declines with age, and if so, Rabbitt's results would represent a conscious or unconscious optimising of strategy to overcome the deficiency. Somewhat similar results, although more pronounced, have been obtained by Brewer and Smith (1984) who compared young retarded adults with normal, and it is plausible to assume a similar explanation in reduced signal-to-noise ratio among retardates.

Fatigue

The decline of performance with time when a maximum muscular effort is continued, is well recognised. Whether similar fatigue effects occur with effortful mental activity has been controversial, but the evidence for them seems clear although they are somewhat subtle and varied. The fact that there have been hardly any studies of fatigue in relation to age perhaps reflects unwillingness to subject older people to tasks that would be unpleasant and possibly dangerous to health, but the practical significance of fatigue, both physical and mental, and its common experiencing by older people, makes the omission serious.

A pioneering attempt by Botwinick and Shock (1952) to study age differences in mental fatigue yielded the, at first sight, surprising result that deterioration in performance of the tasks they used – writing digits or words, addition and digit substitutions, – was *less* among subjects in their 60s than in their 20s. The result is not really surprising. Fatigue decrements occur when performance is carried out at or near the limits of capacity. Botwinick and Shock's subjects were, throughout, slower and could well have been exerting themselves mentally somewhat less than the younger.

How does fatigue affect performance? With neuromuscular fatigue the most noticeable effects are decline of muscular output together with recruitment of muscles not fully loaded, seemingly as an unconscious attempt to assist those that are. The recruitment tends to spread progressively to muscles more and more remote from those immediately concerned, suggesting increased activation which gradually becomes more general. With mental fatigue, several phenomena have been observed (see Welford, 1965b):

- Impairment of visual acuity and lowering of critical flicker frequency.
- Slowing of performance which affects decision times rather than speed of movements.
- Peripheral signals may be missed and less immediately necessary actions omitted.
- Timing may become irregular, so that performance loses "smoothness". In a repetitive task, abnormally long cycles may appear. Such "blocking" has been found to be preceded by a gradual lengthening of cycle time and followed by a return to normal, as if fatigue that had been building up was dissipated during the block.
- Organisation of performance into higher units tends to break down, and established routines may fail.
- In problem solving situations, the short-term retention needed to integrate data to produce a solution may fail so that it takes longer to gain insight into principles involved in the problem.

We do not know to what extent, if any, these changes would, given appropriate tasks, be greater or less in older people, but the worthwhileness of finding out offers an exciting field for future research. The research would seem the more pertinent in that all these fatigue

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changes can be reasonably explained in terms of changed signal-to-noise ratios in the central nervous system, and thus might be expected to interact with age.

Learning and practice

When performance is continued over periods of time but not at an intensity to cause fatigue, it tends to improve progressively. Somewhat arbitrarily, we call the improvement up to the time of substantial mastery of the task *learning*, and subsequent improvement as the effect of *practice*. Studies of learning in relation to age have been summarised by several writers (e.g. Arenberg and Robertson-Tchabo, 1977; Kausler, 1982, 1985; Spilich, 1985) but there are still some serious conflicts of evidence to be resolved and integration of evidence to be done. No attempt will be made here to resolve the conflicts or effect an integration, but some possible leads for the future are given if acquision and retrieval are treated in terms, respectively, of Information Theory and Signal Detection Theory. As regards the former, it seems reasonable to regard any learning task as one of registering information, and to consider the time taken to achieve registration. In these terms the slower acquisition commonly found among older people would be in line with their slower information processing in tasks such as choice reaction times.

As regards retrieval, three types of task need to be considered (Welford, 1985). Let us look first at verbal rote learning, where performance is measured in terms of accuracy of retrieval. It seems axiomatic that if, say, a list of words has to be learnt, the subject's task at the time of retrieval is to discriminate these words from others held in long term memory. In signal detection terms, the normal integral of the number of words correctly retrieved would be expected to increase linearly with the square root of the number of times the words have been presented during the learning trials. This has been found to be so in several sets of experimental results, with the rate of increase for subjects in their 60s and 70s being about 65% of that for subjects in their late teens and 20s. A similar result has been shown in a second type of task – the pursuit rotor – where learning is measured in terms of time on target. In this, learning appeared to take place in two stages: rapidly for the first 7 trials, during which subjects were presumably learning to discriminate the appropriate actions required to perform the task, and more slowly thereafter when they were refining their motor control. Subjects aged 57–86 improved at about 88% of the rate of subjects aged 18–22 during the early trials and at about 80% in the later.

A third type of task is where improvement is not measured in terms of increased accuracy in a given time, but of reduced time for a given accuracy. It has been argued that, in such tasks, discrimination of more from less efficient actions acquired in previous trials "primes" the discrimination at any one trial and thus reduces the time required to trigger accurate action. Results in relation to age with this type of task have been surprisingly variable. When the movements required have been complex and not guided by a display, older subjects appear to have improved more slowly than young. With less complex movements or movements made in response to a display, improvement by both older and younger has been proportional to initial performance. With very simple movements, older subjects have been found to improve faster than younger. It should be added, however, that in no case have they been found to attain equality.

Performance at learning tasks has been found to differ according to whether, at each trial, subjects are or are not required to make a response to each item. If they are, and cannot remember the correct response, they have to guess one on the basis of their general

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past experience. When a guess is wrong, the resulting error tends to persist in subsequent trials to an extent that Kay (1951) said the subjects' main problem in later trials seemed to be to *un*learn the errors they had introduced in the earlier ones. Older subjects, tending to learn more slowly, have to guess more and thus produce more "inventions" (Kausler, 1982, 1985, Spilich, 1985) and more persistent errors (Kay, 1951; Wright, 1957). If, however, it is possible to refrain from responding when unsure, older subjects tend to make more omissions but fewer errors than younger, thus avoiding the danger of persistent errors (Korchin and Basowitz, 1957).

What is learnt? Salthouse and Somberg (1982) suggest that learning may have three effects. One is that subjects come to discriminate relevant from irrelevant information. A second is that they identify relevant and efficient methods of dealing with information. Third, connections from input to output tend to become "built in" and thus made without conscious attention. The effects are not really independent since the second is likely to depend on the first and the third on the second. Salthouse and Somberg found evidence for all three effects in their results, with their older subjects showing less practice effects in each case. They did not find evidence of older subjects performing in qualitatively different ways from the young, but it is only fair to say that the simple tasks they used of signal detection, memory scanning and visual discrimination gave little scope for such differences to be observed.

The use of Signal Detection Theory analysis of learning and practice effects once again illustrates the importance of taking account of errors. For example, it has been shown among young subjects that the degrees of discrimination in signal detection terms for recognition and recall are equal. More items are correctly recognised than recalled, but there are also more false "recognitions" than "recalls". The essential difference between recognition and recall is that the criterion for responding is higher in the latter (Welford, 1986). One wonders whether analysis in these terms could account for at least some of the finding that older people tend to perform relatively better at recognition than at recall.

FOR THE FUTURE

It remains to sum up the implications of what has been said of the past and present, for the future of psychological gerontology. The pioneering work was in the experimental laboratory study of performance, and although over the years research has branched out into other areas such as field observations, psychometric tests and questionnaires, the experimental studies have been central. It is clear that they need to remain so. Field studies were shown early on to need the results of laboratory experiments as guides – without them, the complexity of field situations made it virtually impossible to know what to look for. Psychometric tests and attitude questionnaires need to be validated against actual behaviour and performance, which must again depend on laboratory studies, either directly or *via* field experiments and observations.

The laboratory experimental approach needs, however, to be pursued with caution, avoiding over-dependence on purely psychological theory and the blinkering effects of the hypothetico-deductive method, concentrating instead on an independent assessment of facts. There is also a need to avoid over-dependence on statistical tests: analysis of variance may be an important check on over-enthusiastic interpretation of results, but is no substitute for detailed scrutiny of them. The late G.U. Yule, who was one of the pioneers of statistical methods and a good friend of psychology, once remarked that the use of statistical tests

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was a confession of failure to exercise sufficient experimental control, and that psychology, along with other sciences, should rather aim at the discovery and measurement of functional relationships. A further injunction which used to be customary is still sound: while insisting on scientific rigour in the conduct of experiments, one should not despise the additional insights that can often be obtained by carefully observing subjects while they are performing, and afterwards discussing with them the task and their methods of tackling it.

Apart from these guidelines, psychological gerontology needs to value and enhance the strength it has gained in the past from keeping in close touch with other biological sciences. Most of all, however, there is a need to press constantly towards the attainment of knowledge that can be applied to the practical problems of aging. Not only does this provide a direction and goal without which laboratory research can easily drift into triviality, but it supplies a perspective without which practical problems are likely to be tackled piecemeal without real understanding.

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REVIEW ARTICLE

CANCER AND AGING

A. MACIEIRA-COELHO

Laboratory of Cell Pathology (INSERM), 94804 Villejuif Cedex, France

Abstract—It is widely accepted that the incidence of cancer increases with aging and many hypotheses have been put forward to explain this association. A review of the literature, however, shows that the relationship between cancer and aging has to be reappraised. Although the incidence of most cancers increases during the second half of the animal life span, only with a few does it increase progressively with senescence in humans as well as in animals. As a matter of fact in many cases the incidence does not vary, levels off or even decreases in old individuals. Late developing tumors also seem in many instances to progress more slowly. Thus it seems that the incidence of cancer is related to age rather than to aging and that some conditions may be created during senescence which oppose the development of some cancers.

Key Words: cancer, aging, life span, transformation, cell immortalization, development, carcinogens, oncogenes

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INTRODUCTION

ALTHOUGH THE incidence curve for all cancers combined shows an increase with age in the later half of the human life span, the relationship between senescence and tumor development is rather complex.

Several facts can disturb the investigation of a putative potentiation effect of senescence on the incidence of cancer. When analysing the relationship between a disease and aging, a distinction should be made between age-dependent and senescent-dependent variations in its incidence. The frequency of a disease can increase progressively with age during part of the life span but later decline. In order to be considered as associated with senescence it should increase with aging and should not level off. If incidence curves are not built up to the limit of the animal life span, they can give the impression of a continuous increase which may not exist in reality. Different results can also be obtained depending *inter alia* on the animal species, the animal strain, the type and dose of the carcinogen, the route of administration and the type of tumor. Moreover the use of experimental tumors instead of spontaneous ones to approach the problem, may distort the picture. Indeed the former

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may depend mainly on particular parameters that challenge the host, rather than upon the broad events analysed in epidemiological studies.

Furthermore, what is generally described under the name cancer, corresponds in fact to a variety of pathological conditions where the mechanisms involved in its initiation and evolution vary widely. Thus incidence curves that include all types of cancers do not seem justified. As a matter of fact when one reviews the studies analysing the different types of tumors separately the data raise the possibility that some mechanisms may develop with aging, which not only retard the progression of cancer but also limit its initiation.

INCIDENCE AS A FUNCTION OF AGE

The rate of mortality in the human species doubles every 7 years from 30 to 75, it has been predicted, however, that if cancer could be eliminated at birth or at age 65, life span would only increase by 2.3 and 1.2 years respectively (Hayflick, 1976). This in itself suggests that cancer is not a main event in the aging syndrome.

In humans there are tumors that are typical of childhood, like retinoblastoma, neuroblastoma and Wilm's tumor, and others that are more frequent during adolescence, like osteosarcoma. Other neoplasias can have incidence peaks at 2 different age periods, such as acute lymphocytic leukemia (Cohen *et al.*, 1981) which peaks during childhood, then declines up to age 34 and shows a low and constant increase again from 34 to 60; beyond age 60 the incidence slows down. On the other hand, chronic lymphocytic leukemia and carcinoma of the prostate are typical of people beyond age 50. The frequency of these late developing tumors increases more rapidly with age, peaks around age 75 but then levels off (Lundberg and Berg, 1970; Franks, 1972).

Epidemiology studies in human beings can give quite different results depending on whether they have been collected intra vitam or post mortem. A remarkable cancer morbidity study has been performed in Sweden at the Malmö General Hospital which had an extremely high autopsy frequency (Berge and Lundberg, 1977). These data have been obtained by the systematic examination of all organs during autopsies from all causes of death and thus included even those tumors that remained silent. It also included patients which had been cured from their disease. These studies showed that the incidence of tumors was never above 43% in a human population and that only prostate cancer increased progressively with age and did not level off. The prevalence of all other type of tumors did not increase with aging. Actually the prevalence of most tumors peaked some time during the human life span but then declined. The prevalence of stomach, small intestine and rectum neoplasms for instance, remained the same during the second half of the human life span at all age periods. The prevalence of cancer of the colon in women was steady at all ages; in men it peaked between ages 30 to 34 and 75 to 79, then leveled off. Hence if by definition an aging associated process should increase progressively with senescence, these studies show that cancer cannot be considered as part of the aging syndrome (Pontén, 1977). They also raise the possibility that during aging some mechanisms may develop which can curtail the initiation of cancer and cause the plateauing of the morbidity curves. The plateauing could also mean that those which reach old age are the ones less prone to develop cancer.

In humans those cancers that have incidence peaks earlier, tend to be of the reticuloendothelial system, central nervous system and connective tissues; the others that are more frequent late during the life span are mainly of epithelial origin (Doll, 1978).

The presence of an increased incidence of neoplasia in some premature aging diseases

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such as Werner's Syndrome, has been used as supporting evidence in favor of a relationship between cancer and aging. In Werner's Syndrome however, there is an unusual number of mesenchymal tumors which in the general population tend to appear earlier during the human life span (Salk, 1982). Furthermore, prostatic and pancreatic cancers which are more common in the later part of the human life span, are absent in Werner's Syndrome (Salk, 1982). Hence it is possible that the mechanisms developing during aging, which determine the decline in the morbidity from cancer late during the life span, are more prevalent in Werner's Syndrome patients and cause the absence of those tumors that are typical of the later period but eventually level off in the general population.

The variations in the incidence of cancer according to age also occur in other species. Several types of tumors are characteristic of young rather than old animals (Cotchin, 1975) in particular those caused by a virus such as is the case for leukemias in cat and fowl (Burmester, 1957; Hardy *et al.*, 1973). This shows that the variations in incidence can be related *inter alia*, with the different mechanisms that cause cancer and with the respective response of the host. Young animals seem indeed to be more susceptible to different oncogenic viruses (Bittner, 1936; Gross 1951).

The epidemiology of cancer in species other than the human, has been studied mainly in laboratory animals. In mice there is a very significant increase with age in the incidence of lymphoma and leukemia, for other type of tumors the increase is only slight or there is no increase at all (Ward *et al.*, 1979). In rats the risk of developing an increased number of tumors with age is strain and sex dependent (Burek, 1978). In some strains the animals are at a low risk for all cancers irrespective of age, in others there is a trend for an increased risk with age. Some tumors however, peak at a relatively young age so that older rats are at a much lower risk than the younger animals (Burek, 1978). In the Wistar SPF Tox rat where the tumor incidence is high, this seems to be due to their long life span (Kroes *et al.*, 1981). Hence it is possible that a longer life span allows for a higher incidence, because the prolongation of chronological time also prolongs the time of exposure to carcinogens.

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Epidemiological studies have also been performed in species other than mammals. In the Goldfish for instance, the tumor incidence was proportional to the third power of age (Etoh *et al.*, 1983).

So it seems that in some experimental animals the incidence of tumors is quite different from man; the relationship between histological type and incidence is also different. Tumor epidemiology in experimental animals emphasizes certain parameters associated with a particular strain. They are useful to establish what kind of factors are involved since they render apparent certain determinants which become diluted out when analysing a wild population. The data anyway show, as in studies performed in humans, that senescence does not lead necessarily to an increased tumor incidence.

TIME, DEVELOPMENTAL CHANGES AND CANCER

For those tumors characteristic of the later part of the life span the increased incidence could be due to aging, developmental changes and (or) to a longer exposure to a carcinogen. As mentioned above, the data available from humans as well as from experimental animals, does not favor a simple direct role of senescence in the initiation of cancers.

In regard to the time of exposure to a carcinogen, the results vary widely and show that in some instances the time of exposure to the carcinogen is important; in other instances there is an interplay of additional mechanisms and the time factor becomes irrelevant.

Peto *et al.* (1975) concluded from experiments performed with mice that it is the time of

exposure to the carcinogen that matters since in each group tumors appeared after the same length of exposure and the rate of appearance was unaffected by the age at which exposure began. The epidemiology of bronchial carcinoma in cigarette smokers suggests the same conclusion since the incidence of lung cancer as a function of time is greater in cigarette smokers than in nonsmokers and the incidence in exsmokers remains constant after smoking has stopped (Doll, 1978). Evidence from workers exposed to arsenic however, showed an increasing lung cancer mortality risk with increasing age at initial exposure; the excess mortality was independent of the time since exposure stopped (Brown and Chu, 1983). The incidence of lung cancer caused by another metal (nickel) increased with age at first exposure up to age 25 and then fell off (Doll *et al.*, 1970). It is interesting that the incidence of cancers related to smoking falls off after 80 in cigarette smokers and levels off in nonsmokers (Doll, 1978) again suggesting a possible protective effect of aging. In another study, the relative risk of pulmonary cancer in retired smelter workers was found to decrease with increasing age or time since retirement (Pinto *et al.*, 1978). This could be consistent with a protective effect of aging against neoplastic growth or be due to cohort differences. Another possible explanation is the elimination with time, of transformed cells before they reach full malignant transformation. Data collected after spinal irradiation however, show that the incidence of leukemia and other cancers is proportional to the second and third power of age respectively at the time of irradiation (Doll, 1978).

In those cases where the time of exposure to the carcinogen plays a lesser role, developmental changes that create variations in the susceptibility of tissues to the carcinogens and in the host response to malignant cell transformation could be the main determinant. Indeed Ebbesen (1984) found that the skin of old mice is more susceptible to a chemical carcinogen and to the carcinogenic action of radiation. This was done transplanting old and young skin into young recipients and determining the number of tumors in the grafted tissue. After DMBA treatment, carcinomas developed in 39% of grafts from old donors and in 12% of grafts from young donors. Similar results were obtained with beta radiation. Middle-aged skin however, was not more susceptible to the chemical carcinogen than young skin.

Franks (1979) found that in vitro transformation of bladder epithelium is easier with cultures originated from old than those from young mice; but salivary gland epithelium and mesenchymal cells from different organs showed no effect of age on neoplastic transformation. A review of the literature led indeed to the conclusion that advancing age may involve either an increase, a decrease or no change in the sensitivity of tissues to chemical carcinogens (Anisimov and Turusov, 1981).

The latent period between the action of the carcinogen and tumor formation was studied in rats by Paulini *et al.* (1975). Young animals developed malignant mesenchymal tumors after a mean of 17 months and the old rats after 6 months. In mice no age difference was found in the development of bowel tumors after treatment with 1.2-dimethylhydrazine (Defries *et al.*, 1981).

In summary, these data show that there seems to be an interplay of the type of carcinogens, the time of exposure, and of developmental processes which may either oppose or favor the incidence of cancer.

MECHANISMS INVOLVED IN THE VARIABLE INCIDENCE

One mechanism that could influence the initiation and progression of cancer is variations in the production of growth promoters and inhibitors during the animal life span.

Indeed Ebbesen (1984) found that the senescent skin that was more susceptible to a chemical carcinogen and to radiation, contained less chalone-like activity and responded less well to exogenous chalone.

The identification of the mechanisms leading to a change in the susceptibility of cells to carcinogens with age was approached by Nagasawa and Yanai (1974) measuring ^{3}H-Thymidine (^{3}H-TdR) incorporation in the rat mammary gland at different ages. They found a good correlation between ^{3}H-TdR incorporation and the influence of mammary tumors induced by polycyclic aromatice hydrocarbons.

Sinha and Dao (1980) measured DNA synthesis after administration of DMBA to the rat mammary gland either intravenously or through local applications. With the former route the number of tumors that developed decreased in older rats. Animals 120 days and older were completely refractory to the carcinogen. When DMBA was applied locally all rats developed tumors regardless of age. DNA synthesis was inhibited when the carcinogen was given intravenously but it was stimulated when applied locally. The authors suggested that the local application of DMBA optimizes a tissue carcinogen interaction through the stimulation of DNA synthesis. It was not elucidated if the latter was due to semiconservative synthesis or to DNA repair.

These works show that the rates of cell cycling in tissues through the animal life span, as well as the inducibility of DNA synthesis, seem also to play a role. This is pertinent to the subject dealt with herein since the proliferative activity of some tissues, though not of all, declines with aging.

The affinity of a chemical carcinogen to DNA may also influence the outcome. In the rat, the DNA-binding of DMBA is higher in 50-day-old than 80-day-old animals and is directly correlated with the incidence of tumors (Jauss and Ben, 1978). Changes that occur in the organization of chromatin during cellular senescence (Macieira-Coelho, 1984) could be the cause of the decrease in DNA-binding.

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Carcinogens can be either activated or detoxified by cell enzymatic systems where cytochrome is the more important component. Birnbaum and Baird (1978) measured the levels of cytochrome P-450 content and monoxygenases activity in rats and found that although there is an age dependent decreased level, the senescent rats retained the ability to respond to exogenous inducers. These investigators (Baird and Birnbaum, 1979) also evaluated the generation of mutagenic metabolites of benzopyrene and 2-fluoroenamine by purified hepatic microsomes from rats and mice. The fractions obtained from old animals were more mutagenic as compared to those of young rodents.

The developmental changes occuring in the immune response must also play a crucial role in the incidence of cancer throughout the animal life span. In fact, the relationship between aging, cancer and the immune response has been the object of many hypotheses. Unfortunately our knowledge about the developmental changes of the immune system is still to limited to warrant any speculations in such a complex problem. The relationship between the immunological system and cancer is also far from elucidated. The complexity of the relationship is apparent in the work of Holland *et al.* (1978) who compared survival and cause of death in normal inbred and athymic nude mice. The maximal life span was the same in athymic mice but overall survival rates were reduced due to a higher incidence of lymphoreticular neoplasms. The animals had however, a reduced risk for some solid tumors which according to the authors could explain the greater proportion of athymic mice dying from physiological old age. These experiments show that although senescence in itself may not favor tumor formation, cancer can curtail the animal life span; indeed

the consequence of cancer on overall life span has to be distinguished from the putative relationship between the disease and senescence.

Weindruch *et al.* (1982) like others before, obtained an increased life span and a delayed immune decline in mice kept with a diet providing "undernutrition without malnutrition." They also found a decline in tumor incidence. Since diet in itself is known to play a role in cancer development, this work does not establish a relationship of causality between the improved immune responses and the decreased incidence of tumors. Moreover the results could be due to the use of genetically defined animals with a special pathological pattern. The complexity of the situation is obvious in the work of Ross *et al.* (1983) who concluded that different strains of animals with different spectra of age related diseases could yield different composite models in this type of experiments.

A prolongation of the life span of C3H/Sn mice and a decreased incidence of spontaneous tumors could also be obtained with an immunostimulator obtained from the thymus (Anisimov *et al.*, 1982).

Other events like the production of free radicals have long been proposed to be associated with aging and cancer (Harman, 1962). However, any hypothesis tending to unify these two processes does not seem justified from the review of the literature described herein.

Metabolic changes that occur during development could also influence one way or the other cancer incidence. In addition modifications at a cellular level take place progressively through the animal life span and must also change the susceptibility to carcinogens. They will be discussed below.

TUMOR SPREADING

As mentioned above, post-mortem studies revealed that the morbidity for all human tumors including those that remain silent, peaks around age 75. However, the percentage increase in cancer incidence for England and Wales (Kee, 1983) taken from the number of cancer registries for both sexes, shows a peak between 25 and 30 for women and between 55 and 60 for men. Obviously this study reveals only the cancers giving clinical symptoms but not those remaining silent. It also excluded the early age period since it would distort the picture because of the peak incidence found during childhood. If the incidence of tumors that give clinical symptoms, peaks earlier than that of all tumors including those that remain silent and are only revealed at autopsy, it suggests that at later ages some mechanisms may develop which slow down tumor progression.

This shows the difficulty in establishing the real incidence which should be defined as the age at which the tumor starts. The difference between the time when the tumor starts to develop and the time when it can be diagnosed vary widely for the different types of cancer and can vary for the same type of cancer within patients. Thus the clinical incidence tends to peak later than the real incidence and to underestimate the prevalence of cancer.

Post-mortem studies have the advantage of revealing cancers at an earlier stage, i.e. at least at a time when they become macroscopically visible. Autopsy studies though, to be valid have to cover a long period of time and to be done systematically in all causes of death. They tend to increase the morbidity of cancer in the upper age group since overall mortality increases with age. For obvious reasons they are difficult to perform and have been rarely done meeting all the requirements. To the best of our knowledge the only reliable ones available are those from the Malmö General Hospital (Berg and Lundberg, 1977).

Mortality statistics describe cancer as a cause of death. They do not give a true picture of total incidence since they do not include the cases that are cured or those where "the patient dies with but not from the cancer" (Berg and Lundberg, 1977).

In experimental animals the problem of tumor progression as a function of age has been studied in spontaneous and induced tumors of rats and mice. Progression of spontaneous tumors in rats varies strikingly with the strain. One strain (BL/BL females) had metastatic tumors in 16% of the animals because of the higher frequency of metastatic adrenal cortical carcinomas (Burek, 1978). In this strain there was a positive correlation between age and metastatic cancer. In other strains this evaluation was more difficult to make because of the low incidence of metastatic cancer (3–9% of the animals). Still, the incidence peaked between 24 and 30 months of age and declined thereafter. Burek (1978) concluded that if any trend was seen, it suggested a lack of any increased risk for the oldest rats to develop tumor metastases.

In mice, among late developing tumors, hepatocellular carcinomas metastasized in 12% of animals; other than lymphomas and leukemias few other tumors metastasized (Ward *et al.*, 1979).

Progression has also been studied in experimentally induced tumors. Neoplasias arising in older mice treated with urethane (Anderson and Budinger, 1981) were larger and more invasive. Also, mice with tumors induced by diethylnitrosamine, died earlier with increasing age at the time of treatment (Clapp *et al.*, 1981). Malignant transplanted melanoma grew at the same rate in young and old mice but invasion of the local lymphnode occured earlier in the latter (Hirayama *et al.*, 1984). In these experiments, old mice were also more vulnerable to pulmonary metastases. Opposite results were obtained by another group (Ershler *et al.*, 1984) using the same experimental system. As Hirayama *et al.* (1984) pointed out, the opposite results could be due to differences in the immunogenic and metastatic potential of the melanoma lines, to the differences in inoculum size, site of injection, sex of the animals and time interval of sampling. These differences emphasize the pitfalls that may exist in experimental tumors.

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Dithiotreitol, an immunopotentiator, was used in the same model as an attempt to influence tumor progression. The drug increased the resistance to pulmonary metastases to a greater extent in old than in young mice (Hirayama *et al.*, 1984).

RELATIONSHIP BETWEEN CANCER INCIDENCE AND LIFE SPAN

Cancer, although possibly not a part of the aging syndrome, is one of the diseases that limit the animal life span. Thus, many of those experiments where through manipulation, both the incidence of tumors was decreased and the life span was prolonged, could be due just to the effect of suppressing a cause of death or debilitating disease. In the human species, the population that reaches advanced age and has a decreased incidence of cancer, could be the one less prone to develop cancer and hence more fit to reach the maximum life span. It has been suggested by Walford (1981) that life span is dependent on some genes ("supergenes") that are clustered and regulate broad metabolic events implicated in the development of diseases and in mechanisms that curtail the life span. Genes like those responsible for scavenger enzymes, DNA repair and major histocompatibility antigens (MHC) would belong to this group. An interesting correlation was found between the presence of some MHC genes and the mouse life span (Walford, 1981). A prolonged life span was also associated with a decreased tumor incidence.

An intriguing correlation was found between the life span of some species and the

capacity of the respective cultured fibroblasts to activate a carcinogen into a mutagenic form and to metabolize the carcinogen into water soluble form (Schwartz and Moore, 1977). Moreover a good correlation was found (Schwartz, 1975; Moore and Schwartz, 1978; Pashko and Schwartz, 1982) between species life span and the cytochrome P-448 content of the cultured fibroblasts, which is a major component of the microsomal metabolizing fraction. In other words, shorter lived species have a higher content of cytochrome P-448. These results could favor the possibility raised above, that the life span of some species is curtailed by a higher incidence of tumors which in itself would not necessarily be due to senescence. They could also mean that the same mechanisms involved in the activation of carcinogens and in detoxification are also implicated in senescence. Although it is easier to induce tumors in some of these short lived species (Pontén, 1971), to the best of our knowledge a comparison has not been done between the frequency of spontaneous tumors in short and long lived mammals.

CANCER AND AGING AT THE CELLULAR LEVEL

Germane to the question raised by the works of Schwartz *et al.* (1975, 1977) is the fact that fibroblasts from some short lived species are more easily transformed in vitro than those of long lived ones (Macieira-Coelho et al., 1977a). This is an intriguing phenomenon for which there is no explanation although it has been extensively studied.

It is well known that when one maintains in vitro fibroblasts from different species under serial divisions, the cells from some species lose after a variable number of doublings the capacity to divide (Swim and Parker, 1957; Hayflick and Moorhead, 1961). This has been equated to aging at a cellular level (Hayflick and Moorhead, 1961) and indeed the doubling potential in vitro is inversely related to the age of the donor (Martin *et al.*, 1970). Cells from other species on the other hand, have a high probability of acquiring an unlimited division potential (Hayflick, 1967). When one compares fibroblasts according to the probability of yielding spontaneously a population with unlimited growth potential one finds a scale going from a very low to a 100% probability (Macieira-Coelho *et al.*, 1977a). The latter is found with mouse fibroblasts regardless of the animal strain. In general murine fibroblasts tend to have a higher probability of yielding permanent cell lines (cell immortalization) than for instance human and bovine. Pertinent to the problem dealt with herein, is the fact that it is easier to induce cancers in those species whose fibroblasts have a higher probability of escaping senescence (Pontén, 1971).

Immortalization is one of the steps in the complex phenomenon called transformation which is the counterpart in vitro of malignancy in vivo (Hayflick, 1967). Transformation consists in the progressive acquisition of growth autonomy, such that a cell population becomes less dependent upon the different mechanisms which restrain the growth of normal cells in vitro. During transformation a cell population goes through a series of steps whose succession is unpredictable and which can stop short of full autonomy. Transformation of chicken fibroblasts by Rous sarcoma virus (RSV) for instance, yields cells which go through some transformation steps (e.g. piling up and loss of contact inhibition of growth) but which do not immortalize and indeed have a shorter life span than the non-infected cells (Pontén, 1971). Tumors developed in chicken after RSV infection also do not have a clonal type of growth (Pontén, 1971). The spread of these tumors is due to increased cell proliferation after viral infection; the infected cells die after a few divisions and the virus is propagated to new cells which thus become recruited into the proliferative

compartment (Pontén, 1971). Pertinent to these mechanisms of transformation and malignancy is the fact that chicken fibroblasts belong to that type of cells with a low probability of spontaneous immortalization.

It is interesting that the response to carcinogens and to oncogenes in vitro seems to be related to the proneness to senescence or spontaneous immortalization of the different type of fibroblasts. Indeed, while rodent cells can be easily immortalized by chemical, viral and physical carcinogens (Pontén, 1971) the probability for this event to occur with human fibroblasts is low (Macieira-Coelho *et al.*, 1976; Namba *et al.* 1981). This is also true for oncogenes: the probability of transforming human fibroblasts this way seems extremely low (Sager *et al.*, 1983). On the other hand, experiments on the transforming potential of oncogenes have been mainly performed on rodent cells which anyway transform spontaneously (Cooper and Neimen, 1981; Land et al., 1983). Hence aging and transformation at the cellular level seem to be two opposing forces.

Another finding showing that transformation and aging at the cellular level represent two opposing forces, is the fact that the division potential of fibroblasts from some cancer patients does not decline with the age of the donor, as is the case for the cells of normal donors (Azzarone *et al.*, 1984). The fibroblasts from the cancer patients also show in vitro other deviations from normalcy suggesting that they are less influenced by the mechanisms that restrict the growth of normal cells in vitro (Azzarone *et al.*, 1984). It has been suggested that this is due to the left over of an embryonic phenotype in these mesenchymal cells with the creation of a field effect that facilitates the transformation of epithelial cells (Shor *et al.*, 1985).

Several experiments suggest possible explanations for these different behaviors of cells in vitro. Some of them concern observations at the cytogenetic level during serial divisions of these cell populations. They suggest that the long term doubling potential of cells is directly related with the potential for continuous rearrangements, in other words, with the plasticity of the genome.

Human embryonic fibroblasts when cultivated in vitro go through continuous chromosomal rearrangements without any definite pattern becoming predominant (Chen and Ruddle, 1974; Harnden *et al.*, 1976). The data suggested that multiple clones arise continuously and compete between each other without anyone overgrowing the others. These cells have a longer division potential than postnatal fibroblasts which go through more stable clonal type chromosomal rearrangements during serial divisions in vitro (Harnden *et al.*,1976).

Another finding pertinent to this concept is the fact that fibroblasts from Werner's Syndrome patients, which have a reduced doubling potential when compared with cells from age-matched normal donors, present chromosomal rearrangements (variegated translocation mosaicism) which become predominant and remain so during the cell population in vitro life span (Salk *et al.*, 1981). All these findings suggest that a "rigidity" of the genome is associated with a shorter doubling potential as opposite to a higher plasticity which would lead to a prolonged cell life span.

It is also interesting that sister chromatid exchanges in human fibroblasts which have used most of their doubling capacity, are less stimulated by drugs known to induce them (Schneider and Gilman, 1979). This finding suggests that the loss of the division potential is associated with a loss of the plasticity of the genome.

Furthermore human embryonic fibroblasts when irradiated with low dose rate radiation at low population doubling levels (PDL) are more resistant and their life span can even be

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prolonged (Macieira-Coelho *et al.*, 1977b). When irradiated late during their life span however, their doubling potential is shortened. The same type of radiation shortens the division potential of postnatal fibroblasts, and it seems that these different effects are related to the "plasticity" or the "rigidity" of the cell genome (Bourgeois *et al.*, 1981). On the other hand this type of radiation can prolong the life span of human postnatal fibroblasts from donors with genetic diseases with a high rate of chromosome recombinations, which are at a high risk of cancer (Diatloff and Macieira-Coelho, 1979).

Low dose rate ionizing radiation can also accelerate the acquisition of an infinite growth potential in mouse fibroblasts in vitro (Macieira-Coelho *et al.*, 1976) a cell whose genome seems to be endowed with a high degree of plasticity. Indeed additional evidence in favor of an association between genome plasticity and prolonged division potential comes from observation on some characteristics of the mouse cell genome. The chromosomes of mouse fibroblasts cultivated in vitro show a very unusual capacity of recombination; images of crossingover and bridges between chromosomes suggesting interchromosomal exchanges can be observed with a high frequency (Macieira-Coelho, 1980). Furthermore the chromatin labile sites which accumulate during cellular aging (Beaupain *et al.*, 1980) are eliminated in the case of mouse cells during these chromosmal rearrangements that occur during the transition from senescence to immortalization. This plasticity of the mouse genome could well be at the basis of the propensity to escape senescence at the cellular level and to progress in the path of transformation. It is also probably due to this plasticity that in the wild, different mouse subspecies are found with different chromosome sets (Capanna, 1973).

These features led to the hypothesis that during cell division a reorganization of the genome takes place (Macieira-Coelho, 1980). This reorganization can be accelerated by viruses, chemicals and radiation. The outcome depends on characteristics of the cell genome which will favor or not survival after the recombinational events inevitable during the division cycle. Depending on the interaction of these properties of the cell genome, the reorganization will lead either to cellular senescence or to a progressive autonomy. Considerable evidence has accumulated showing that indeed a reorganization of the genome takes place at the chromosomal and molecular level during cell division (Macieira-Coelho, 1984). From the data reviewed above, a relationship between the potential for reorganization at the cytogenetic level and the long-term division potential seems to exist. It remains now to elucidate the characteristics of the genome at the molecular level which allow a cell to escape from senescence and proceed in the path to transformation.

Another question that can be raised at the cellular level is if the susceptibility to carcinogens varies during aging in vitro. To the best of our knowledge there is only one reference on this subject, where it is claimed that it is easier to transform aged human fibroblasts with SV40 virus (Girardi *et al.*, 1965). It would be interesting to confirm these results and to check the response to other viruses and other carcinogens.

CONCLUSION

An analysis of the literature concerning the relationship between cancer and age, reveals the complexity of the disease. The number of parameters that can influence this relationship is almost unlimited. It is obvious that although the incidence of most cancers tends to increase during the second half of the human life span, a dissociation between cancer and aging becomes apparent in many instances.

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Incidence peaks for different types of cancer can be found through the whole life span. One possible explanation for this variable incidence is that the main determinant factor may vary for the different types of tumors. Cancer can be caused by different agents and one agent may play a more important role than the others for the triggering of certain types of tumors and also be more important at given ages.

Neoplasia is the result of multiple changes occuring in a cell through the action of a variety of agents, and it also depends on the state of the cell. In this respect genetic determinants can play a role in early developing tumors (e.g. retinoblastoma) as well as late developing ones (e.g. mammary cancer) (Anderson, 1972). An important finding concerning the state of the cell is the one showing that at least in some cases neoplasia seems to be a generalized disease which becomes expressed mainly in a specific organ (Azzarone *et al.*, 1984). Indeed the apparently normal somatic cells of cancer patients when cultivated in vitro, manifest many properties which represent deviations from normalcy. This was found in patients with different ages, becomes apparent long before the detection of the tumor (Azzarone *et al.*, 1984) and could be due to inborn or acquired cellular properties which create a favourable background for the initiation of neoplasia.

Developmental modifications proceeding through the animal life span are certainly important for the variable incidence of cancer. At the tissue as well as the cellular level a permanent remodeling goes on from birth through maturity and to old age. This continuous modifications can be favorable at a certain time for the initiation of some types of cancer and can create later adverse conditions for malignant cell transformation. Metabolic modifications such as the production of hormones and growth factors, are another example of the variables that can influence the incidence of cancer through the animal life span.

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The response of the host to foreign antigens and to modifications of its own antigens, through the immune system, is another important parameter. The senescent related changes in the immune response may not favor inexorably the incidence of cancer. Some of the works reviewed above suggest that the physiologic remodeling going on in the immune system may at some time be favourable for the development of some neoplasias but later protect the organism against other tumors.

The variable incidence as a function of age depends also on the carcinogen and the way it reaches the cell. This is quite obvious from the examples cited above where results varied depending on the route of administration. Moreover while the factor time may be important for some carcinogens it may be irrelevant for others. In many instances the incidence of chemical carcinogen related cancers, declines later during the life span.

Finally all these parameters do not play a role only in the triggering of neoplasia but also in its progression. As described elegantly by Foulds (1958) the development of neoplasia in an organ is not necessarily followed by a relentless invasion of the tissue and later metastases. Tumor progression can stop for an unpredictable period of time or may even be completely arrested until the animal dies of old age without any obvious cause of death. Progression can also proceed by intermittent steps and then stop or else the tumor can finally spread and kill the host. Hence one can find an unlimited type of situations during cancer initiation and progression. As far as aging is concerned the data suggest that late developing tumors in general have a slower growth.

The thought of a simple direct correlation between both cancer and senescence is not suggested from a comprehensive analysis of the literature. On the contrary it seems possible that some mechanisms develop during senescence which limit cancer initiation and

slow down its progression. At the moment one can only say that cancer is in part related to developmental processes and if an association exists at all with senescence, it concerns only a few types of tumors.

A better understanding of the mechanisms implicated in the variable incidence of cancer could provide a more rational approach to the prevention and the cure of the disease.

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IS A FAT METABOLITE THE MAJOR DIET DEPENDENT ACCELERATOR OF AGING?

C.J.I. DRIVER*, R. WALLIS and G. COSOPODIOTIS

Victoria College, Rusden Campus, 662 Blackburn Rd, Clayton, Victoria 3168, Australia

G. ETTERSHANK

Dept. Zoology, Monash University, Clayton, Victoria 3168, Australia

Abstract—Previous work with a high fat/low carbohydrate diet indicated that *Drosophila melanogaster* aged faster on that diet than on a high carbohydrate/low fat diet. This could be due to one of eight possibilities enumerated. Oxygen consumption of flies on the high fat/low carbohydrate diet is initially the same as that on the control diet. Subsequently the oxygen consumption falls to below control levels at older ages. No increased rate of living is indicated. A starvation resistant strain is described. The male eats less food, and yet maintains a body fat level indistinguishable from the parent strain. The lifespan and most physiological indices of aging used indicate that the process of aging is unchanged. Nutrient dilution produces a nearly compensatory increase in food intake with a reduced body fat and body energy reserves. The indices of aging used indicate that the rate of aging is reduced under these circumstances. These results are consistent with the possibility that fat or a fat metabolite alters the rate of aging. It is argued from previous data that body fat itself is not likely to be the important factor, rather a metabolite is probably involved.

Key Words: Drosophila, aging, diet, fat, metabolite

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INTRODUCTION

IT WAS reported previously that the lifespan of *Drosophila melanogaster* was shortened on a high fat/low carbohydrate diet (Driver and Cosopodiotis, 1979). Several dietary fats were tested for their effects on longevity and all of these were found to shorten the lifespan substantially. Some of these lipids, especially the unsaturated fats were so effective as to suggest toxicity. However the replacement of glucose in the diet with palmitic acid produced a modest reduction in lifespan which subsequent work suggested was partly due to an accelerated aging. First, return of the flies from the high fat diet to the low fat diet showed an irreversible shortening of subsequent lifespan compared to flies that had been fed the low fat diet for the same time, and this shortening was increased by increasing the time on the high fat diet (Driver and Cosopodiotis,1979). Second, in the flies fed

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this high fat diet the irreversible deterioration of a number of organ systems occurred more rapidly than on the low fat diet (Driver, 1984). The possibility could be argued that Drosophila are adapted to a high carbohydrate diet and therefore would be unable to survive as well on a diet for which there is no metabolic apparatus. In fact, surprisingly little is known about what the flies eat in the wild. Nevertheless Drosophila adapt to a wide variety of diets in the laboratory and the flies are quite capable of surviving quite well on the high fat diet used in this study. The high fat diet used in this series of experiments is not devoid of carbohydrate, in fact substantial amounts of starch are present in the cornmeal, although data presented by Driver (1984) indicates that undegraded starch may appear in the feces, indicating that all the starch is not available. Other evidence suggests that this diet is adequate by several criteria. Eight generations of flies have been raised on the high fat medium without loss of viability as judged by the numbers, size, and sex ratio of the emergent adults of the next generation (unpublished data). Furthermore, as reported in Driver and Lamb (1980), when adults without previous exposure to the test diet were fed a high fat diet for a short period of 7–10 days, their glycogen reserves were unaffected and the fat reserves were only slightly reduced. At the same time there was little change in the amount of food being consumed. During this time they eat their own weight of medium (calculated from the data of Driver and Lamb, 1980.) and would be expected to develop serious energy imbalances on an indigestible diet. On the other hand with advancing age the flies energy stores fall, especially on the high fat diet, and this could well contribute to the early death observed on the high fat diet.

The acceleration of aging observed on this diet could be due to one of several factors including the energy deficit mentioned above. For effective nutritional prophylaxis of aging it is important to define the crucial factor(s) more closely. Therefore to differentiate between these several possibilities, two other situations were examined, using a range of criteria similar to that previously used. The nutrient density of the medium was reduced, and the effects on physiological decline and longevity were observed. In addition a starvation resistant strain is described. Despite significant differences in food intake, lifespan and the physiological indices of decline were unchanged. Comparison of these results with those published previously allow us to suggest that the data is most consistent with a metabolite of fat being the significant factor that links nutrition and aging.

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MATERIALS AND METHODS

Details of media and culture are described elsewhere by Driver and Cosopodiotis (1979) and Driver and Lamb (1980). Male Canton S flies were used. The control medium contained glucose (25g), cornmeal (45g), dried yeast (12g), agar (9g), methyl 4-hydroxybenzoate (2g), and water (700 g). For 25% and 50% dilutions the nutrient components glucose, cornmeal and yeast were reduced by 25% and 50% respectively. For the high fat/low carbohydrate diet, palmitic acid (10g) was substituted for the 25g of glucose in order to produce an isoenergetic replacement. The yeast added to the medium was boiled with the rest of the mixture and were therefore killed. No live yeast was added to the medium.

For oxygen consumption measurements, flies were kept in air in a 20 cc syringe at 25°C for 4–6 h and the oxygen concentration determined by injecting the gas through a filter into a Beckman model E2 oxygen analyser.

Locomotory behavior and grooming behavior were measured by direct observation as

described previously (Driver, 1984). The flies were kept in a controlled temperature cabinet at 25°C so that the activity measured refers to activity in the environment in which lifespan was measured. The activity measured was not spontaneous activity as the intermittent vibration of the cabinet acted as a stimulus.

The rise in food intake was estimated by determining the density of fecal pellets as described by Driver and Lamb (1980).

Body fat was determined by the difference between the dry masses before and after extraction with petroleum ether (40°–60°) as described previously (Driver and Lamb, 1980).

Where indicated, irreversible components of change were differentiated by transferring groups of flies from one medium to another at weekly intervals up to five weeks. Results were then analysed statistically to give the reversible and irreversible, cumulative effects of the medium on that parameter as previously described (Driver, 1984).

For starvation selection, groups of 100 flies segregated into sexes were placed in vials containing cotton wool dampened with distilled water and capped with a foam plastic stopper. They were then incubated until approximately 10% survived in each sex, rescued by transferring to a vial with normal medium, and then bred. This procedure was repeated three times with successive generations to produce the starvation resistant strain.

Drosophila age pigment was measured by the method of Ettershank, Macdonnell and Croft (1983), modified as follows. Groups of 20 flies were killed by freezing and stored frozen for later analysis. They were then immersed in chloroform (1 cc) and sonicated for 2 min at 100W. Another 1 cc of chloroform and 1 cc 100 mM $MgCl_2$ was added with stirring. The mixture was allowed to stand and the supernatant was drawn off and discarded. The remaining solution was extracted three times more with 1cc 100 mM $MgCl_2$ and the mixture centrifuged at 4°C. The fluorescence was then determined according to Dillard and Tappel (1971) at 470nm and using 400nm as an excitation wavelength. Quinine sulfate (1mM) in 1M sulfuric acid was used as a calibration standard.

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RESULTS

Oxygen consumption

To test for the possibility that the rate of living might be increased on the high fat/low carbohydrate diet the oxygen consumption was measured in flies of three different ages. These ages were chosen because at 21 days the flies on the high fat diet had reached the end of the plateau phase in their lifespan and at 41 days the flies on the low fat/high carbohydrate diet reached the end of the plateau phase of their life table. It is worth pointing out that considerable differences were observed between experiments in oxygen uptake, presumably due to variation in locomotory activity. Although valid comparisons within the experiment were possible since the results are consistent from one experiment to another, these measurements show poor agreement with the results other authors. Our results are shown in Fig. 1. There is no difference initially in the oxygen uptake, but at 41 days on the high fat diet, the flies were using less oxygen. This difference may be at least partly due to selective enrichment of more viable, genetically different flies on the high fat/low carbohydrate diet and therefore the difference may be partly artifactual. Nevertheless there is no elevation of the oxygen uptake as would be predicted by the rate of living model.

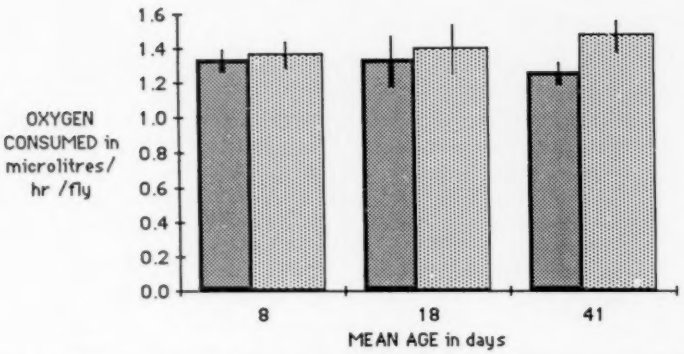

FIG. 1. Oxygen consumption of flies on a high fat diet (dark areas) or on a low fat diet (light areas). Measurements were performed on 10 groups of 20 flies for each group represented as columns. Standard errors of measurements are shown as bars on top of each column. The difference between the values at 41 days is significant ($P < 0.01$).

Starvation resistant strain males

Starvation resistant males excreted less than the parent strain indicating that they ate less. In addition they were more active than the parent strain. In spite of this they maintained very nearly identical body fat, indicating a more energetically efficient metabolism. These results are summarised in Fig. 2.

The average lifespan is unaltered in this strain. In addition the spontaneous activity deteriorates in parallel with the control strain, and the increase in grooming frequency changes in parallel with the parent strain. By contrast the rate of accumulation of age pigment is slower. These results are summarised in Fig. 3.

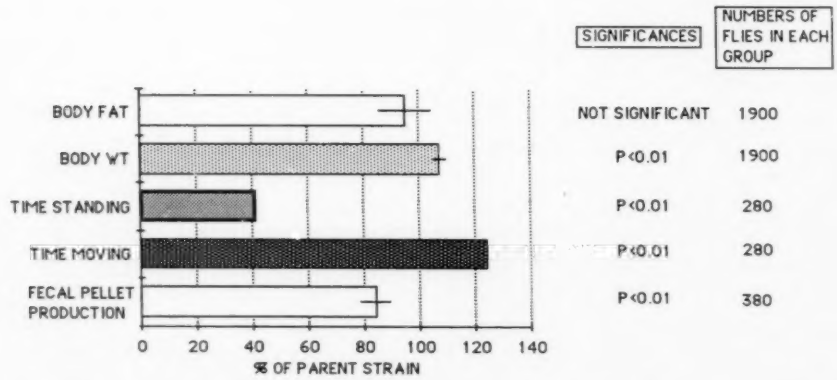

FIG. 2. Some physiological values of the starvation resistant strain compared with the parent strain. The significance of the differences of the time spent in standing or moving were determined as the differences in proportions. The significances of the differences of the other values were determined by the Student's test. The standard errors of the differences (where applicable) are shown as lines on top of the bars. The numbers of flies used in the comparisons and the significance of the differences are listed to the right of the bars.

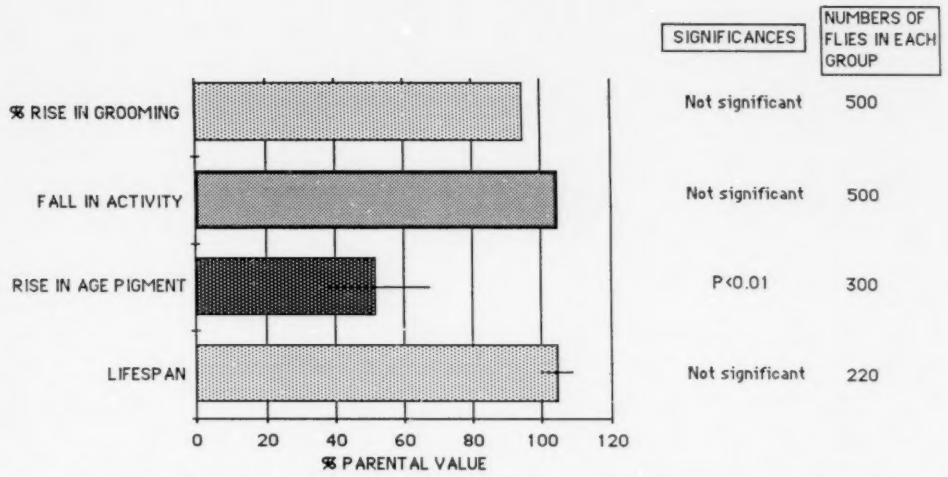

FIG. 3. Comparison of the aging indices of the starvation resistant strain with those of the parent strain. The significance of the differences were determined by the difference of proportions test in the first two data sets. In the second two date sets, the Student's t test was used. The standard errors of the differences are shown as lines on the bars. The significance of the differences are shown on the right of each bar, together with the numbers used in each group for each comparison.

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Medium dilution

Dilution of the medium nutrients produces a compensatory increase in food intake as determined by fecal output (Fig. 4). In spite of this, body energy reserves are reduced as measured by body fat and starvation survival time (Fig. 5).

Dilution of this type produces a modest, variable increase in lifespan if instituted on young adults, but a reduction in lifespan if initiated at a later stage in their life. This indicates that a chronic degeneration process is retarded if medium dilution is initiated early

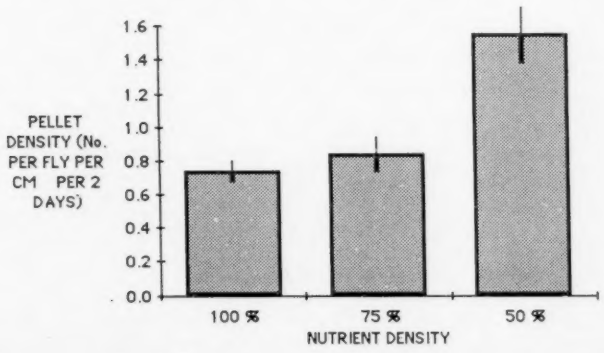

FIG. 4. Fecal production and nutrient density. The rises shown are significant ($P < 0.01$) and are not significantly different from the predicted from a response that would give equivalent energy intakes. Standard errors of the means are shown as lines on top of the bars. The measurements were on 4-day-old flies with 320 flies in each group.

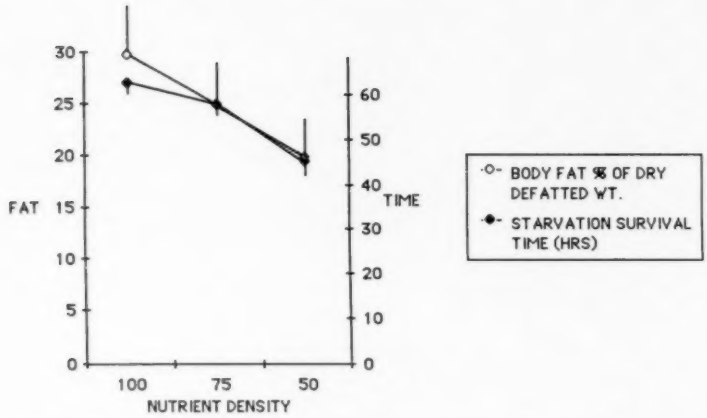

FIG. 5. The effect of energy density of the medium on energy reserves. Approximately 120 flies were used in each group. The standard error of the measurements are shown as bars above or below the experimental points. There is a significant downward slope for each set of points ($P < 0.01$).

enough, but if introduced late in life the deleterious effect of energy shortage may be a more important effect (Fig. 6).

Other physiological declines and pathological changes were also measured. Dilution reduced the rate of accumulation of age pigment (Fig. 7) as well as the rate of change of spontaneous activity, an effect that was not reversed by transfer back to the control medium (Fig. 8). Furthermore, dilution reduced the rate of change of grooming, partly irreversibly (Fig. 9). On the other hand the rise in pellet production (after correction for the immediate response to food dilution) increases more rapidly and irreversibly (Fig. 10).

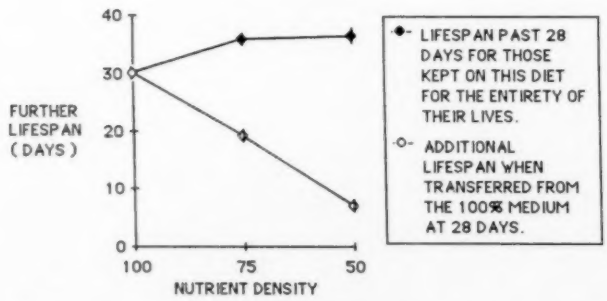

FIG. 6. The effect of medium dilution on further survival past 28 days. The top curve shows the effect of medium dilution for flies fed the medium for their entire life. The bottom curve shows lifespans for flies kept on a control diet until 28 days and then put on the diluted media. Between 100 and 250 flies were used in each group, and the standard error of the means are shown on the points as vertical lines. All differences are significant ($P < 0.01$).

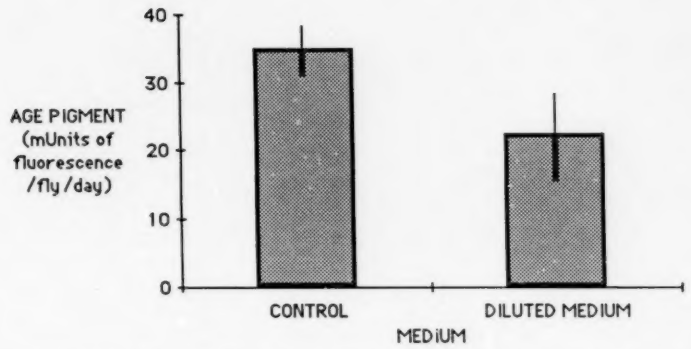

FIG. 7. Age pigment increases with time on two media. Standard errors of the means are shown as lines on the bars. The difference is significant ($P < 0.01$).

DISCUSSION

Flies kept on a high fat diet for their entire lives have a shorter lifespan associated with an increased rate of aging. In a nutritional experiment in which one diet is compared with another, it is not possible to ascribe the differences observed to one factor because several changes are made that might be relevant. In this case it has been possible to enumerate eight major groups of factors that might be important in the increased rate of aging.

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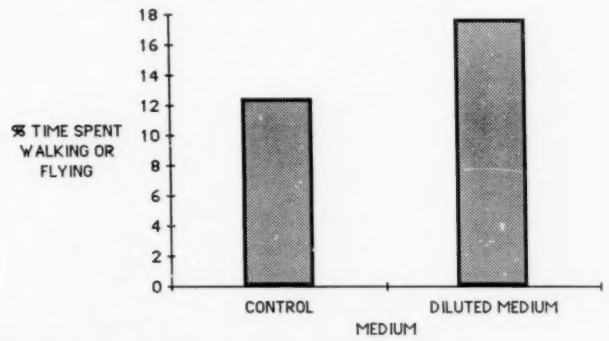

FIG. 8. Locomotory activity on the two media after six weeks. Ten groups of flies were used with 145 in each group. Five observations were performed on each fly at six weeks. Five groups were fed initially on each medium and at one, two, three, four, and five weeks one group was transferred from one medium to the other. Analysis of activity of each of these groups allowed not only a comparison of the locomotory activity if maintained on each medium but also to what extent the differences could be reversed by transferring to the other medium. The fall in activity on the diluted medium is less than on the control medium ($P < 0.01$), using the significance of differences proportion test. Furthermore these differences are cumulative and irreversible using the significance of the regression line for the values obtained for all groups ($P < 0.01$).

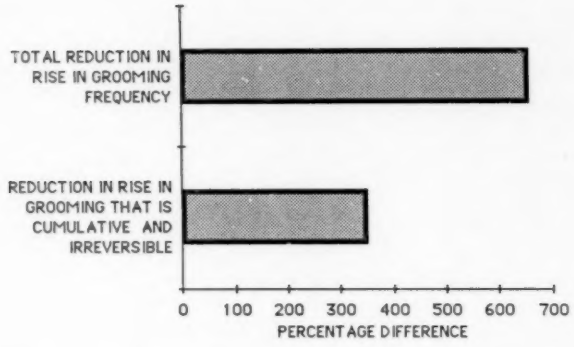
TOTAL REDUCTION IN RISE IN GROOMING FREQUENCY
REDUCTION IN RISE IN GROOMING THAT IS CUMULATIVE AND IRREVERSIBLE
0 100 200 300 400 500 600 700
PERCENTAGE DIFFERENCE

FIG. 9. Rise in grooming frequencies after six weeks on the two media. Numbers, experimental design and calculation of significances were as on Fig. 8. The difference is significant ($P < 0.01$). Part of the difference is reversible (47%, $P < 0.01$). The irreversible component of the differences is cumulative ($P < 0.01$).

First possibility: rate of living model

Data from the high fat/low carbohydrate fed flies. When Drosophila were kept at a higher temperature, their lifespan was shorter than those kept at a lower temperature. This observation led a number of authors to postulate a rate-of-living hypothesis that still continues to stimulate discussion and experimentation. According to this hypothesis high temperatures increase the "rate of living" and a higher rate of living shortens the lifespan by using up "vitality" more rapidly. Additional support for this theory arose from a number of experiments including those showing that food restriction may prolong lifespan under some circumstances. These and other observations relating to the rate-of-living are reviewed by Lamb (1977).

In the first set of experiments the possibility was considered that the rate of living model could be invoked to explain the difference. To rationalise the model it was necessary to

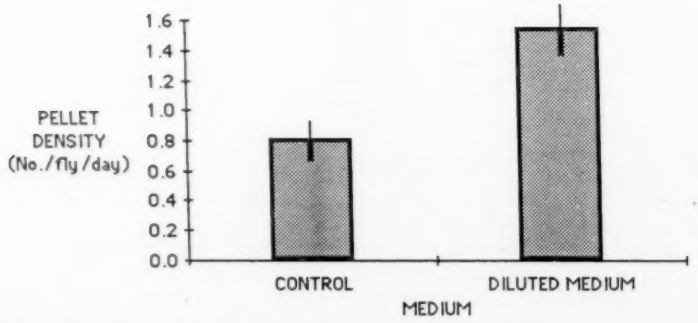

FIG. 10. Comparison of the rise in fecal pellet density for the flies kept on the two media. For this experiment 280 tubes of vials of flies containing 10 flies each were used. Irreversible components of the changes and significances were determined as detailed on Fig. 8. The differences are significant ($P < 0.001$), cumulative and irreversible ($P < 0.001$).

suppose that the rate of respiration on the high fat diet was higher, possibly because of a uncoupling of mitochondrial respiration from ATP production by unesterified fatty acids. However there was no increased rate of respiration in either old or young animals. Therefore this possible explanation is not applicable. Furthermore the starvation resistant strain appears to be more active than the control strain and yet the lifespan is unchanged, and the rate of deterioration of most indices tested is unchanged. This too suggests that the rate of living model is not correct.

The starvation resistant strain. The starvation resistant strain eats less than does the parent strain, is more active and retains the same level of body fat. This suggests that they may be more energy efficient than the parent strain. The lifespan is unaltered, and most physiological indices of aging measured were unaltered, suggesting that the aging rate is unaltered.

In this strain, the level of body fat remains identical to that of the parent strain in spite of the reduced intake of food. As the rate of aging remains unaltered it appears that the intake of food materials such as carbohydrates, proteins or most micronutrients may be unimportant as determinants of aging. On the other hand fat and fat related metabolites remain a possible factor in age determination.

Medium dilution. Most indices of aging are reduced by long term feeding of diluted medium, and the lifespan is increased suggesting that the rate of aging is reduced. On the other hand the deterioration of metabolic efficiency is accelerated, and if this is important in the death of flies then this may explain why dilution of medium is not always beneficial.

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When the flies are presented with a diluted medium the intake of food is increased to compensate approximately for the reduced nutrient density. Nevertheless body fat levels remain reduced. The rate of aging is apparently reduced in this situation, in spite of the fact that the intake of nutrients is not appreciably altered. Thus fat (or a metabolite) remains a plausible candidate for the nutrient related substance that accelerates aging.

Second possibility: the total intake of food

On a high diet the older flies eat more food per day (Driver and Lamb, 1980) and therefore it could be argued that the increased food intake accelerates aging. This proposal would be consistent with the data on medium dilution, but is not consistent with the data on the starvation resistant strain where the rate of aging is unchanged in spite of a reduced intake of food.

Third and fourth possibilities: the intake of carbohydrate or another nutrient is reduced

It is possible that the lower levels of carbohydrate on the high fat/low carbohydrate diet might be the age accelerating factor. Alternatively a metal ion may complex with fatty acids and become unavailable, and aging becomes accelerated because of the lack of that nutrient. In either case it would be expected that a reduced intake of food would be expected to produce an increased rate of aging. In fact in these studies a reduced intake of food is associated with no change in the starvation resistant strain, and a reduced of aging on diluted medium. These results are not compatible with the possibility that the absence of carbohydrate or fatty acid complexed nutrient is an important factor.

Fifth possibility: uptake of a nutrient is increased by fatty acids

It is possible that the free fatty acid present in the high fat medium may enhance the uptake of a fat nutrient that accelerates aging. It this were so then a reduced intake of food that is low in fat might be expected to reduce the rate of aging in the first case. However it is found that reduced intake of food is associated with no change in the rate of aging in the starvation resistant strain.

Sixth possibility: energy shortage

Older flies on a high fat diet experience a greater energy deficit (Driver and Lamb, 1980) and therefore the possibility exists that this energy shortage might be imprortant in accelerating aging. However the flies on the diluted medium appear to be also energy deficient while they have a reduced rate of aging. This indicates that an energy shortage itself is not important in accelerating aging.

Seventh possibility: effects of microflora

In these experiments as is the case with many published works on diet the test organism has a number of associated bacteria and possibly eukaryotic organisms. To minimise this uncertainty no live yeast were added to the medium. Methyl 4-hydoxybenzoate, a bacteriocide and fungicide, was added to the medium and the more osmotically active sugar glucose was used instead of the more commonly used sugar sucrose. In fact visible colonies of bacteria or fungi were very rarely found and when they were found the tube of flies and the results relating to them were discarded. Nevertheless in these flies the gut commonly became colonised with bacteria in middle age. The possibility that these might make a difference in the lifespan was discounted because the starvation resistant strain has a much lower incidence of infection and yet has a lifespan that is unchanged. Conceivably there could be a role for microflora in the acceleration of aging on the high fat/low carbohydrate diet, but in the light of the above arguments it would probably be a metabolite of fat and not the fat itself.

Eighth possibility: fat or a metabolite

The remaining possibility is that fat itself or a metabolite is an accelerator of aging. The data in this paper are consistent with this conclusion. In the starvation restrain strain there is no change in aging and no change in body fat. A reduced rate of aging occurs when the medium nutrients are diluted. However, it cannot be body fat itself that is important, because an increased rate of aging in Drosophila is associated with a reduction in body fat on a high fat diet (Driver and Cosopodiotis, 1979; Driver and Lamb, 1980; Driver, 1984). On the other hand the data could well be consistent with substances such as lipoproteins, or a bacterial metabolite as the determining factors. While this process of elimination fails to show what is the important substance, the number of likely possibilities is reduced.

In what way might fat or its metabolites produce irreversible damage? Many possibilities exist. For example, free radicals may be generated during normal metabolism of fat since some of the fat is desaturated by a microsomal enzyme system. In addition the metabolism of fat by peroxisomes generates significant amounts of hydrogen peroxide, a potential source of highly reactive oxygen radicals. Other mechanisms are possible and as yet there is no evidence that fat exerts its effects through a free radical mechanism. Fur-

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ther information on other diets, and other species, particularly in sterile diets may add considerably to the understanding of the nature of the putative agent and the mechanism of action. Furthermore it would seem that investigations into agents that modify fat metabolism may prove worthwhile.

Acknowledgments—The authors would like to thank Professor B. Cragg (retired) for his help in preparation of microscope slides; Mrs. J Russel, Ms. K. Patterson and Ms. M. Paull for their assistance with preparation of media, and Dr. R. Gay[illegible] for his invaluable criticism of the manuscript.

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EFFECT OF EXPERIMENTALLY-PROLONGED LIFE SPAN ON FLIGHT PERFORMANCE OF HOUSEFLIES

R.S. SOHAL and J.H. RUNNELS

Department of Biology, Southern Methodist University, Dallas, Texas 75275

Abstract—Reduction of metabolic rate by the elimination of flying activity extends the life span of male houseflies about two-fold as compared to those permitted to engage in flying activity. Flying performance of flies is known to undergo an age-associated decline. The objective of this study was to determine if such an age-related decline in flight performance is delayed by the reduction of metabolic rate. Flight performance was measured, by cross-sectional sampling at different ages, by the "stationary flight" method, as the total time spent in flight and the number and duration of individual flights and rest stops during a one hour observation period. Flies kept under conditions of low physical activity exhibited a superior flight performance at all ages as compared to those kept under high activity conditions. The decline in flight performance observed in old flies was delayed in the longer-lived low activity flies as compared to the shorter-lived high activity flies. Results support the concept that the rate of aging in houseflies is modified by variations in the level of physical activity.

Key Words: aging, life span, physical activity, flight, housefly

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INTRODUCTION

ONE OF the most ubiquitous senescent alterations in virtually all species is the decline in locomotor activity. In the flying insects, flight performance, as measured by stamina, distance flown and speed of flying have been reported to decline in old insects (for review see Baker, 1976; Sohal, 1976, 1985). In the housefly, Rockstein and Bhatnagar (1986) have reported that the duration of induced flight decreases steadily with age.

Previous studies in this laboratory have indicted that the rate of metabolism is inversely related to the life span of adult houseflies, e.g. the average and the maximum life expectancy of flies is extended almost two-fold by elimination of flying activity (Ragland and Sohal, 1973; Sohal and Allen, 1986). In addition, the rate of a variety of age-related cellular and biochemical changes was found to be retarded by the decrease in metabolic rate of the flies (for review, see Sohal, 1986; Sohal and Allen, 1986). Results of these studies have led us to propose the hypothesis that the rate of aging of the organisms is linked to their rate of metabolism (Sohal, 1981, 1986; Sohal and Allen, 1985, 1986).

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If metabolic rate influences the rate of aging and if decline in flight performance is truly a manifestation of senescence, it will be expected that manipulations of metabolic rate of flies would affect the expression of the age-related changes in flight activity. The specific objective of the present study was to test this hypothesis by determining whether or not the age-related decline in the flight performance of the housefly is ameliorated by the decrease in the rate of metabolism of the flies. Metabolic rate was affected by variation of flying activity. Flight performance was measured as total duration of flying and as the number and the duration of individual flights and rest stops during one hour observation periods.

MATERIALS AND METHODS

Rearing of Houseflies

Eggs obtained from 7 to 10-day old flies were placed on moist CSMA (Chemical Specialties Manufacturers' Association) fly larval medium. After emergence from the pupae, the adult flies were segregated by sex, and fed on sucrose and water. The ambient temperature was maintained at 25°C and relative humidity at 45%.

Variation of Physical Activity

The level of physical activity and hence of metabolic rate was varied by altering the flying activity of the flies. To achieve relatively high levels of physical activity, 200 male flies were housed in each one-cubic foot cage, where they were able to fly. To reduce physical activity, houseflies were confined singly in a urine specimen glass bottle (about 250ml), partitioned with a cardboard maze in such a manner that flies could walk but were unable to undertake flight due to limitation of space. Individual confinement is necessary to reduce the level of physical activity because in a group, male flies persistently engage in homosexual copulatory attempts which increases their physical activity and shortens their life span (Ragland and Sohal, 1973; Sohal and Buchan, 1981). We have previously shown that the rate of oxygen consumption of flies kept in large cages is significantly higher than those kept in the small bottles (Sohal *et al.*, 1984).

Comparison of Flight Performance

Flight performance of the flies was measured by the "fixed flight" method described by Rockstein and Bhatnager (1966) and depicted in Fig. 1. An inverted J-shaped slender cop-

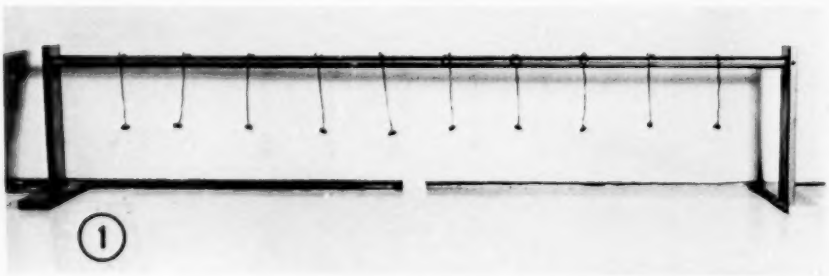

FIG. 1. Photograph of the experimental set up for the measurement of flight performance by the "fixed flight" method.

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per wire was attached to the dorsal mid-thoracic region of the fly using Super Glue®. The other end of the wire was secured to a suspended heavy metal rod. Most of the flies thus suspended usually undertook flight without any stimulus from the experimentalist. Flight performance was measured by cross-sectional sampling of flies at different ages, as the total time spent in flying, average number and duration of individual flights and rest stops during a one hour period, following which the flies were killed.

RESULTS

Mortality characteristics of the flies under conditions of high and low levels of physical activity have been reported before (Ragland and Sohal, 1973; Sohal *et al.*, 1984). In a typical experiment the average life expectancy of houseflies was 19.5 ± 6.7 days under conditions of relatively high physical activity (HA) and 33.3 ± 14.7 days low activity (LA) conditions, whereas, the maximum life spans in two groups were 28 and 65 days, respectively.

A certain proportion of flies in both groups did not engage in flight activity under the experimental conditions (Table 1). The proportion of flies willing to undertake flight decreased with age in the LA group, especially in the latter part of life. In HA group, declining trend was noticeable only between 4 to 14 days of age. At all comparable ages a relatively larger percentage of LA flies undertook flight as compared to HA flies.

In both the LA and the HA group, the overall flight performance of the flies declined in the old flies near the time of achievement of average life span. However, this decline occurred at a later age in the LA group than in the HA group. In the HA group, the total time spent in flight during the one-hour observation period increased between 2 to 4 days of age and declined somewhat by 7 days of age, remaining at the same level in 14-day old flies and followed by a notable decrease in the 21-day old flies (Fig. 2). Whereas the frequency of flights in the HA group tended to increase with age, the average duration of a flight declined after 4 days of age.

In the LA group, the measurements on flight performance were initiated at 7 days of

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TABLE 1. PERCENTAGE OF FLIES UNDERTAKING FLYING ACTIVITY AT DIFFERENT AGES UNDER THE EXPERIMENTAL CONDITIONS.

	Percent flies undertaking flight	
Age (days)	*High Activity Group*[a]	*Low Activity Group*[b]
2	65 (43)	-
4	88 (45)	-
7	77 (48)	100 (49)
14	51 (35)	97 (39)
21	71 (45)	90 (54)
28	-	76 (46)

[a]Numbers in parenthesis represent the total number of flies used in the test.

[b]Measurements of flight performance were begun at 7 days of age in order to allow the effects of low physical activity to be manifest.

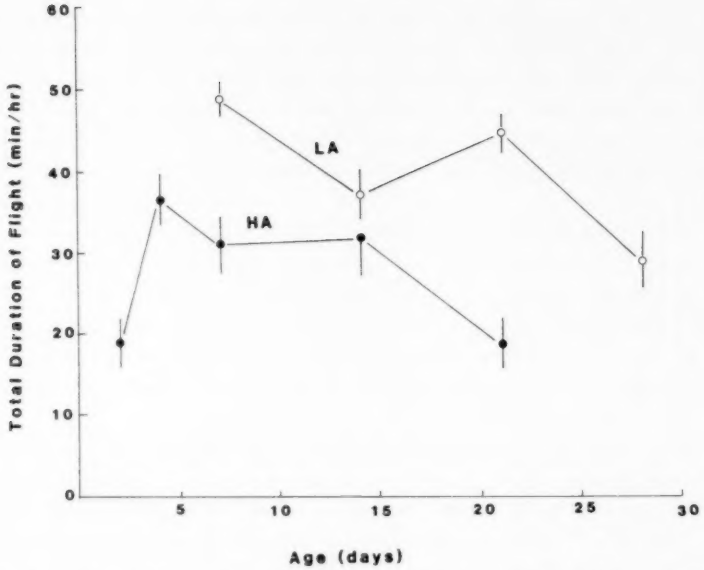

FIG. 2. Comparison of the total duration of flying in flies, kept under conditions of relatively high (HA) and low (LA) levels of physical activity at different ages, during a one hr observation priod. N = 28, 40, 37, 18 and 32 in 2-, 4-, 7-, 14- and 21-day old HA flies, respectively and 49, 38, 49 and 35 in 7-, 14-, 21- and 28-day old LA flies, respectively. Values are average ± SEM.

age because it was felt that the effects of the experimental regime would not accrue until a certain length of time had elapsed. The studies in the LA flies were extended to 28 days of age because these flies had a considerably longer life span than the HA flies. At all comparable ages, LA flies spent greater time in flight than the HA flies. The total flight duration in the LA flies remained relatively high until 21 days of age, but declined notably at

TABLE 2. THE AVERAGE NUMBER AND LENGTH OF FLIGHTS AND REST STOPS IN HOUSEFLIES OF DIFFERENT AGES KEPT UNDER CONDITIONS OF HIGH (HA) AND LOW (LA) LEVELS OF PHYSICAL ACTIVITY DURING A ONE HOUR OBSERVATION PERIOD.

Age (days)	*Group*	*Number of flights*	*Length of flights (min)*	*Number of rest stops*	*Length of rest stops (min)*
2	HA	2.0 ± 0.3	9.3 ± 2.0	2.3 ± 0.3	17.8 ± 2.6
4	HA	2.2 ± 0.3	16.5 ± 2.2	1.9 ± 0.3	12.1 ± 2.1
7	HA	2.5 ± 0.4	12.6 ± 2.0	2.2 ± 0.4	12.8 ± 2.1
	LA	1.3 ± 0.1	38.6 ± 3.1	0.8 ± 0.3	12.7 ± 2.4
14	HA	4.3 ± 0.7	7.7 ± 1.3	3.8 ± 0.7	7.1 ± 1.6
	LA	1.9 ± 0.2	19.5 ± 2.6	1.4 ± 0.3	16.8 ± 2.7
21	HA	3.3 ± 0.4	5.7 ± 0.8	3.5 ± 0.4	7.1 ± 1.6
	LA	2.4 ± 0.3	19.0 ± 2.1	1.8 ± 0.3	8.4 ± 1.2
28	LA	3.9 ± 0.4	7.6 ± 1.1	3.7 ± 0.4	8.2 ± 0.9

Note: Values are average ± SEM. Some of the data on HA flies was reported previously in a different context (see, *Arch. Geront. Geriatr.* **5**: 183, 1986)

28 days of age. The average number of flights increased with age but the average length of a flight decreased 50% between 7 and 14 days of age; remained at a comparable level between 14 to 21 and sharply decreased between 21 to 28 days of age.

A comparison of the average number and duration of flights and of average number and duration of rest stops between HA and LA flies is presented in Table 2. At all comparable ages, flies of the LA group undertook fewer flights but of longer duration than the HA flies. The LA flies also took fewer rest stops than the HA flies. The length of the rest stops in the two groups was comparable at 7 and 21 days of age, but at 14 days of age they were longer in LA flies than in HA flies. However, at the latter age the average number of rest stops taken by LA flies were only 37% of those taken by HA flies. In general, the flight performance of 14-day old HA flies was most comparable to the 28-day old LA flies.

DISCUSSION

Results of this study differ from those reported by Rockstein and Bhatnager (1966) in the housefly. Contrary to their finding, the duration of flight did not decrease linearly with time starting at one day of age. Following an initial developmentally-related improvement, flight performance remained at a relatively high plateau, albeit with some minor age-related changes, until a sharp decline occurs towards the end of life. It has been noted previously that there is considerable individual variation among cohort insects in their propensity to fly as well as in duration of flight under experimental conditions (Davis, 1980). Although presently little is known about insect psychology, and insects are often believed to respond only instinctively, it is conceivable that housing conditions may affect the flight behavior of insects. However, there is no relevant evidence in this area to guide us. In the present study, the percentage of flies that were willing to engage in flight under experimental conditions was greater among those kept under conditions of low physical activity where flying was not permitted as compared to those kept under conditions allowing flight activity. It is therefore possible that the propensity for flight may be a variable factor in the high- and low-activity groups. In spite of this complication, the flies of the LA group exhibited a superior flight performance at all ages and in most respects the parameters of flight performance of 14-day old HA flies were similar to those of 28-day old LA flies. Because the life span of LA flies is nearly twice as long as that of the HA flies, flies at these ages can be considered to be of similar "physiological" age.

Previous studies exploring the possible causes of age-related decline in flight performance in dipterans have correlated flight loss with substrate levels, enzyme activities, mitochrondrial respiratory efficiency as well as protein synthesis rates (for review see, Rockstein and Miquel, 1973; Chesky, 1985). In particular, a correlation between flight performance and glycogen reserves has been reported in drosophila (Williams *et al.*, 1943) and several different species of mosquitoes (Rowley and Graham, 1968; Nayar and Sauerman, 1973). In addition, the old mosquitoes were reported to be unable to fully utilize the available glycogen reserves during exhaustive flight (Rowley and Graham, 1968).

It has been previously shown in this laboratory that the complete loss of flight ability, which precedes death in all aged houseflies, occurs earlier in flies undergoing higher levels of physical activity as compared to those kept under low activity conditions (Ragland and Sohal, 1973). Results of this study indicate that the overall age-related pattern of flight characteristics, such as the average number and duration of flights, is similar in HA and LA flies, but is expressed over a longer period in the latter group. Altogether, results of this and previous studies suggest that the age-related decline in flight ability is delayed

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under conditions of relatively low metabolic rate and are consistent with the view that experimental decrease in metabolic rate of flies retards their rate of aging.

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EFFECT OF AGE ON PARATHYROID HORMONE AND FORSKOLIN STIMULATED ADENYLATE CYCLASE AND PROTEIN KINASE ACTIVITY IN THE RENAL CORTEX

H. JAMES ARMBRECHT, MONICA A. BOLTZ and LEONARD R. FORTE

Geriatric Research, Education and Clinical Center, Veterans Administration Medical Center, Departments of Internal Medicine and Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63125
and
Harry S. Truman Memorial Veterans Administration Hospital, Department of Pharmacology, University of Missouri School of Medicine, Columbia, Missouri 65201

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Abstract—The capacity of parathyroid hormone (PTH) to stimulate the renal production of 1,25-dihydroxyvitamin D declines with age. Since the action of PTH in the kidney is mediated by cAMP, we have examined the effect of PTH and forskolin on renal cortical adenylate cyclase in young (3 months), adult (13–15 months), and old (25–27 months) F344 rats. PTH-dependent adenylate cyclase, measured as cAMP accumulation in cortical slices, was reduced in adult and old rats compared to young rats over a PTH concentration range of 0.015–15 units/ml. There was no difference in PTH-dependent adenylate cyclase activity between adult and old rats. Renal plasma membrane preparations demonstrated similar changes in PTH-stimulated adenylate cyclase activity. There was no difference in calcitonin, forskolin, or guanyl-5-ylimidodiphosphate (Gpp(NH)p) stimulation of adenylate cyclase in plasma membranes from each age group, suggesting that the defect lies in the membrane receptor for PTH. The decreased adult sensitivity to PTH could be reversed by thyroparathyroidectomy. PTH stimulation of cytosolic protein kinase activity did not change with age. These results suggest that the decrease in PTH-dependent adenylate cyclase is due to the alterations at the level of PTH receptor. These alterations may be in response to the increase in serum PTH seen in these animals with increasing age.

Key Words: age, parathyroid hormone, forskolin, adenylate cyclase, protein kinase, kidney, receptors

INTRODUCTION

THE ACTION of many hormones on their target tissues changes with age (Florini and Regan, 1985). However, in most cases the specific biochemical alteration responsible for the altered tissue response is not known. A critical step in the activation of vitamin D is the conversion of 25-hydroxyvitamin D (25(OH)D) to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the most biologically active form of vitamin D, by the kidney (Deluca,

Correspondence to: Dr. H.J. Armbrecht, Geriatric Center (111G-JB), VA Medical Center, St. Louis, MO 63125.
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1979). This conversion is under tight hormonal regulation and is markedly stimulated by parathyroid hormone (PTH) in the young. However, the capacity of PTH to enhance the renal production of $1,25(OH)_2D$ declines with age in both rats (Armbrecht *et al.*, 1982) and man (Tsai *et al.*, 1984).

There is good evidence that PTH exerts its effects in the kidney by increasing intracellular levels of cAMP. Dose-response and time-course studies are consistent with the involvement of cAMP in the stimulation of renal $1,25(OH)_2D$ production by PTH (Armbrecht *et al.*, 1984b). In addition, forskolin, which markedly stimulates adenylate cyclase (AC) at a post-receptor site, also stimulates renal $1,25(OH)_2D$ production (Armbrecht *et al.*, 1984a).

Since the effects of PTH are mediated by cAMP, we have examined the effect of age on PTH-stimulated renal AC activity over the lifespan of the rat. As reported previously in 2 and 12-month-old rats (Marcus and Gonzales, 1982), we have confirmed that there are maturational changes in the PTH-stimulated accumulation of cAMP by renal slices. We have extended these observations by studying the AC activity of isolated renal plasma membranes from young, adult, and old rats. Forskolin and a GTP analog were used to determine the probable site of the change. Serum immunoreactive PTH and calcitonin was measured for possible correlation with changes in AC activity. Finally, we looked for possible age-related changes in PTH-stimulated protein kinase activity.

MATERIALS AND METHODS

Male Fischer 344 rats aged 3 (young), 13–15 (adult), and 25–27 (old) months were purchased from Charles River Breeding Laboratories, Wilmington, Mass. The 13 and 25-month-old rats were retired breeders. The Fischer 344 rat has a 50% mean survival of 29 months under microbial barrier conditions (Coleman *et al.*, 1977). Serum creatinine, an index of renal function, does not change with age in these rats (Coleman *et al.*, 1977). Rats were fed a vitamin D sufficient diet containing 1.2% calcium and 0.8% phosphorus throughout their lifespan. In some experiments, rats were thyroparathyroidectomized (TPTX) to remove endogenous PTH. Surgery was performed under sodium pentobarbital anesthesia, and rats were killed 72 h after surgery. TPTX was verified by a decrease in serum calcium of greater than 2 mg/100 ml.

Agonist-stimulated cAMP accumulation was measured in renal slices as previously described (Armbrecht *et al.*, 1984b). Briefly, thin slices of renal cortex were preincubated for 15 min in 1.0 ml Krebs-Ringer bicarbonate buffer and then 20 min in buffer containing lmM 1-methyl-3-isobutylxanthine (MIX), a cyclic nucleotide phosphodiesterase inhibitor. During the final 5 min of the second incubation, agonists were added to the slice incubation medium. For cAMP determination, slices were homogenized in 0.5 ml 50 mM sodium acetate buffer, pH 4.0, and heated at 95°C for 10 min. The homogenate was centrifuged, and the supernate was assayed for cAMP using the protein-binding method (Gilman, 1970). Tissue content of cAMP was expressed as picomoles per milligram wet weight of tissue. Under these incubation conditions, the tissue content of cAMP reflects tissue AC activity because MIX has been shown to completely inhibit cyclic nucleotide phosphodiesterase activity (Zenser *et al.*, 1977).

The effect of agonists on protein kinase activity was also measured using renal slices (DeRubertis and Craven, 1976). Slices were incubated as described above for cAMP accumulation. At the end of the incubation, slices were homogenized in cold buffer, the

homogenate was centrifuged, and the protein kinase activity of the supernate was determined on the same day. The capacity of the supernate to phosphorylate calf thymus histone was determined in the presence and absence of 2 μM cAMP (Corbin and Reimann, 1975). Protein kinase activity ratios were calculated as the ratio of the protein kinase activity obtained in the absence of cAMP to that obtained in the presence of cAMP.

Agonist-stimulated AC activity was measured in renal plasma membranes. Plasma membranes from renal cortex were isolated by differential centrifugation (Fitzpatrick *et al.*, 1969). The plasma membrane preparations obtained from this procedure were characterized using the enzyme marker Na/K-ATPase (Fitzpatrick *et al.*, 1969). The specific activity of Na/K-ATPase in the homogenate did not change with age (22.0 ± 2.8 and 20.6 ± 1.5 nmol/min/mg for young and old rats, respectively). Likewise, the enrichment of Na/K-ATPase in the plasma membranes relative to the homogenate did not change significantly with age (14.3 ± 2.6 and 22.9 ± 7.4). The effect of agonists on plasma membrane AC activity was measured using ^{32}P-ATP (Herman *et al.*, 1978). The ^{32}P-cAMP product was isolated using a two-step column procedure (Salomon *et al.*, 1974). Recovery of the cAMP product was 40–50%. Activity was expressed per miligram of membrane protein (Lowry *et al.*, 1951).

In some experiments, blood was collected and serum calcium, phosphorus, immunoreactive PTH (iPTH), and immunoreactive CT (iCT) were determined. Serum calcium was determined using a fluorometric technique (Kepner and Hercules, 1963), and phosphorus was measured colorometricly (Fiske and Subbarow, 1925). Serum iPTH was measured using a nonequilibrium radioimmunoassay (Conway and Anast, 1975). The standard curve used in these experiments was made by dilution of pooled sera from vitamin D-deficient rats containing a high level of iPTH. Chicken antibovine PTH (CH977) was used as antiserum, and this antiserum exhibits the highest binding affinity for the midregion of the bovine PTH (1–84) molecule. The lower limit of assay detection was 1.4 μl eq/ml. Serum iCT was measured by radioimmunoassay using goat antibody to human CT and using human CT as standards (Anast *et al.*, 1975). The lower limit of detectability of this assay was 100 pg/ml.

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PTH used in these experiments was synthetic bovine PTH (1–34) (6800 I.U./mg) purchased from Beckman (Palo Alto, Calif.). PTH was dissolved in 150 mM NaCl (pH 3.9). CT was salmon calcitonin purchased from Armour Pharmaceutical (Kankakee, Ill.) and dissolved in isotonic saline (200 I.U./ml). Forskolin was purchased from Calbiochem-Behring (LaJolla, Calif.), dissolved in ethanol, and added from a concentrated stock solution. The nonhydrolyzable GTP analogue guanyl-5-ylimidodiphosphate (Gpp(NH)p) was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.) and dissolved in buffer.

The data from these experiments are reported as the means ± standard error (S.E.). Statistical analyses were performed using Student's two-tailed t-test (Dixon and Massey, 1969), and a confidence level of 95% or greater was considered significant.

RESULTS

The effect of age on PTH-stimulated AC activity was studied using renal slices (Fig. 1). PTH increased cAMP accumulation in a dose-dependent manner in all age groups over the concentration range studied (0.005–15 units/ml). However, PTH-stimulated cAMP production was significantly lower in renal slices from adult and old rats compared to young

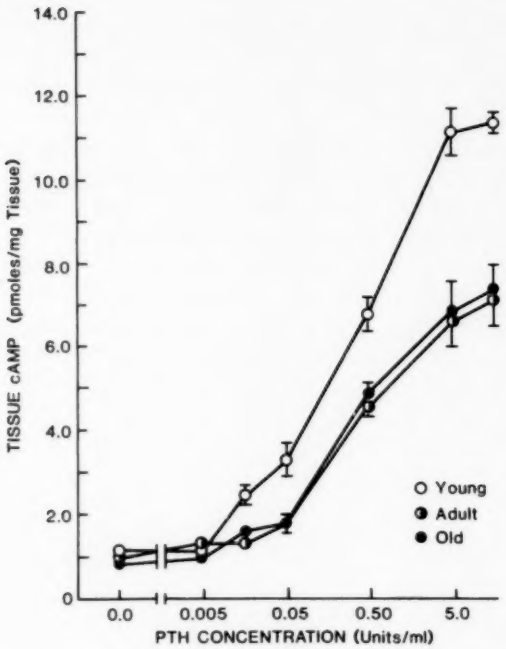

FIG. 1. Effect of age on PTH-stimulated accumulation of cAMP by renal slices. Renal slices from rats of each age group were incubated 5 min with the indicated concentration of PTH in the presence of 1 mM MIX. Slices were then extracted and assayed for cAMP. Data points are the mean ± S.E. of 5 slice incubations.

rats in the PTH concentration range of 0.015–15 units/ml. PTH-stimulation of renal slices from old rats was not significantly different from that of the adult. There was no significant change in basal levels of tissue cAMP (no PTH) with age.

To determine whether the age-related decline in tissue cAMP was intrinsic to the membrane-bound enzyme complex, AC activity was measured in renal cortical membranes (Table 1). Concentrations of agonist which gave maximum responsiveness were used. AC activity in untreated (Control) membrane did not change significantly with age.

TABLE 1. EFFECT OF AGE ON ADENYLATE CYCLASE ACTIVITY OF RENAL CORTICAL MEMBRANES*

	Adenylate Cyclase Activity (pmoles cAMP/10 min/mg)		
Agonist	*Young*	*Adult*	*Old*
Control	164 ± 16	188 ± 15	203 ± 22
PTH (15 units/ml)	868 ± 16	454 ± 49†	486 ± 54†
Forskolin (100 μM)	2950 ± 243	2774 ± 164	3145 ± 248
Gpp(NH)p (100 μM)	500 ± 66	533 ± 41	506 ± 23
Calcitonin (10 units/ml)	604 ± 7	772 ± 37†	749 ± 74

*Table entries are the mean ± S.E. of 10 (Control) or 3 to 4 (others) replicates.
†Significantly different from Young of same treatment group ($P < 0.05$, t-test).

TABLE 2. EFFECT OF AGE ON SERUM Ca, P, iPTH AND iCT*

Age	Serum Ca (mg/100 ml)	Serum P (mg/100 ml)	Serum iPTH (μl equivalents/ml)	Serum iCT (pg/ml)
Young	10.5 ± 0.8	8.05 ± 0.40	17 ± 11	150 ± 14
Adult	10.9 ± 0.5	5.71 ± 0.26†	95 ± 29†	717 ± 45†
Old	10.6 ± 0.5	5.42 ± 0.21†	150 ± 20†	459 ± 50†

*Table entries are the mean ± S.E. of 5 to 7 animals.
†Significantly different from Young ($P < 0.05$).

In the presence of PTH, AC activity in adult and old rats was only 52–56% of that seen in membranes from young rats. Compared to control, PTH stimulated AC activity 5.3 fold in the young, but only 2.4 fold in membranes from adult and old rats. These results are similar to those obtained in slices (Fig. 1).

In contrast to PTH, AC activity in the presence of forskoiin, Gpp(NH)p, and calcitonin did not decrease with age (Table 1). Forskolin greatly increased AC activity 15–18 fold in membranes from all age groups. Gpp(NH)p stimulated activity 2.5–3.1 fold, and this activity did not decline with age. Calcitonin stimulated activity 3.7–4.1 fold, and there was actually an increase in activity in the adult group compared to the young group.

The serum concentration of calcium, phosphorus, iPTH, and iCT was determined as a function of age in some experiments (Table 2). There was no change in serum calcium with age, but serum phosphorus was significantly lower in the adult and old groups compared to the young group. Serum iPTH was significantly higher in the adult and old groups despite the lack of change in serum calcium. There was no significant difference between the serum phosphorus and iPTH of the adult and old rats. Serum iCT was also significantly elevated in the adult and old groups compared to the young group.

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Hyperparathyroidism is known to decrease PTH-sensitive AC activity in the kidney (Carnes *et al.*, 1978). Therefore, adult rats were TPTXed to determine the effect of removal of PTH on PTH-stimulated AC. AC responsiveness to PTH and forskolin was then measured using renal slices (Table 3). In the presence of PTH, the TPTXed adult rats demonstrated AC activity that was significantly higher than that seen in the nonTPTX rats and comparable to that of the young rat (7.30 ± 0.50 pmoles/mg in the same experiment). Forskolin-stimulated AC activity, on the other hand, was not significantly increased in TPTX rats compared to nonTPTX adult rats. TPTX had no effect on PTH-stimulated AC activity in the young rat (data not shown).

TABLE 3. EFFECT OF THYROPARATHYROIDECTOMY ON PTH-AND FORSKOLIN-STIMULATED ADENYLATE CYCLASE ACTIVITY*

	TISSUE cAMP (pmoles/mg wet weight tissue)		
Condition	Control	PTH (5 units/ml)	Forskolin (100 μM)
SHAM	1.29 ± 0.10	5.57 ± 0.42	18.1 ± 1.5
TPTX	1.44 ± 0.12	7.70 ± 0.48†	19.5 ± 1.7

*Table entries are the mean ± S.E. of 10 to 12 slices from 5 to 6 adult animals.
†Significantly different from SHAM ($P < 0.05$).

Since differences in PTH-stimulated AC activity were demonstrable in renal slices from each age group, parallel experiments were conducted to determine the effect of PTH on protein kinase activity (Fig. 2). There were no significant differences in basal protein kinase ratio in the absence of PTH with age, although a trend toward increased ratios with age was observed. There were also no significant age differences in protein kinase activity at each PTH concentration tested (Fig. 2). In the young, the ratio was significantly increased over basal in the range of 0.005–5.0 units/ml PTH. In renal slices from adult and old rats, no significant increase over basal was seen until a concentration of 0.50 units/ml PTH due to slightly higher basal ratios in these groups. In all age groups, changes in the protein kinase ratio were due to changes in the protein kinase activity in the absence of cAMP. There was no change in total activity of protein kinase activity in the presence of cAMP with age (39.0 ± 2.7, 42.1 ± 2.9, and 40.6 ± 3.5 pmoles/min/mg protein in young, adult, and old age groups, respectively).

DISCUSSION

These results demonstrate that there is a decrease in the capacity of PTH to raise intracellular cAMP levels in the kidney with age. This change takes place early in the

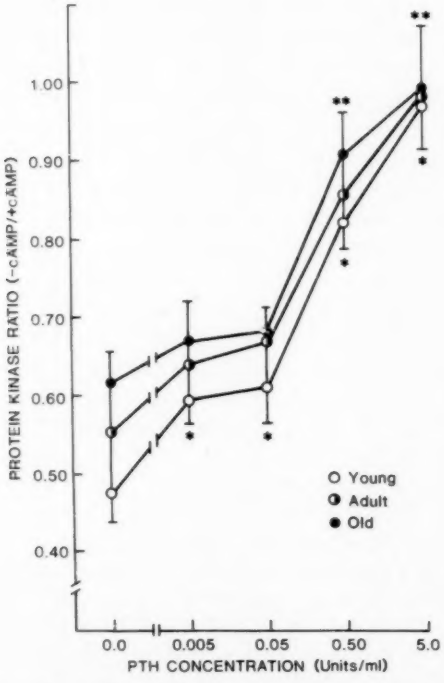

FIG. 2. Effect of age on PTH-stimulated protein kinase activity. Experimental procedure of Fig. 1 was followed and protein kinase activity of the cytosol was measured. Activity was expressed as the ratio of activity measured in the absence (−cAMP) and presence (+cAMP) of 2 μM cAMP. Data points are the mean ± S.E. of 8 incubations. Single asterisk indicates significant ($P < 0.05$, t-test) stimulation of young slices by PTH above young control (no PTH). Double asterisk indicates significant stimulation of adult slices and old slices by PTH above control.

lifespan, since there is a large decrease between the young and adult animals, and there is no further decrease between the adult and old animals. This decreased capacity to raise intracellular cAMP is due to a decline in the PTH-stimulated AC activity found in the plasma membrane. This decline in activity is specific for PTH since there is no decrease in basal or CT-stimulated AC activity with age.

Several lines of evidence suggest that this decline in PTH-dependent AC is due primarily to alterations in the PTH receptor in response to the changes in serum PTH with age. First, PTH-stimulated AC decreased as serum iPTH increased. The greatest increase in serum iPTH was seen between the young and adult rat compared to the increase between the adult and old rat (Table 2). The greatest decline in PTH-stimulated AC activity was also seen between young and adult rats (Fig. 1). Second, removal of the thyroparathyroid glands increased PTH-stimulated AC activity in adult rats (Table 3). Third, there was no age-related decline in forskolin and Gpp(NH)p-stimulated AC in membrane preparations (Table 1). Forskolin acts at the catalytic subunit (Martin *et al.*, 1984), and Gpp(NH)p acts at the nucleotide regulatory subunit to stimulate cAMP production (Ross *et al.*, 1983). The fact that there is no age-related change in the action of these compounds suggests that the changes in PTH-stimulated AC activity are at the level of the PTH receptor. However, changes in other components with age cannot be completely ruled out by these experiments. Decreased receptor number without an apparent change in receptor affinity in response to hyperparathyroidism has been demonstrated in the chick (Forte *et al.*, 1982).

In contrast to PTH, CT-stimulated AC showed no decline with age and was actually higher in the adult compared to the young (Table 1). This is further evidence that the age defect in PTH-stimulated AC lies with the PTH receptor and is not the result of changes in the regulatory or catalytic subunits with age. However, it should be noted that PTH and CT stimulate different cell types along the nephron (Morel, 1983). CT-dependent AC showed no age-related changes despite the increases in serum iCT that were as large as the increases in serum iPTH (Table 2). It has been reported that the hypocalcemic effect of CT declines with age (Copp and Kuczerpa, 1968), but the mechanism of this decline has not been elucidated.

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The changes in PTH-stimulated AC activity (Fig. 1) did not result in a decline in cytosolic PTH-stimulated protein kinase activity with age (Fig. 2). This may reflect the fact that the renal protein kinase is activated by very low levels of cAMP (Armbrecht *et al.*, 1984b). At each PTH concentration, the activity ratio was actually higher in the older animals compared to the younger. This may reflect the higher basal levels of protein kinase activity in the older animals. A similar increase in basal protein kinase activity with age has been reported in the liver (Montgomery *et al.*, 1982). Although there was no change in total cytosolic activity with age in the kidney, these experiments do not rule out age-related changes in specific subcellular pools and/or types of protein kinase.

A decrease in agonist-sensitive AC between young and adult rats is not unique to the kidney but has also been demonstrated in other tissues. Studies have demonstrated an age-related decline in beta-adrenergic-stimulated AC in rat erythrocytes (Bylund *et al.*, 1977) and human lymphocytes (Krall *et al.*, 1981) and in dopamine-stimulated AC in the rat striatum (Puri and Volicer, 1977). The decline in agonist-sensitive AC in erythrocytes and the striatum are correlated with a decline in receptor binding. However, it is not known whether this loss of receptor binding is in response to increased exposure to agonist with age or to some other mechanism. In human lymphocytes, receptor binding does not change with age (Halper *et al.*, 1984), but the stimulation of AC by GTP analogs and NaF

does change (Krall *et al.*, 1981). This suggests a post-receptor defect. In contrast, the decrease in PTH-stimulated AC in the kidney appears to be due to changes at the receptor level in response to increased PTH levels.

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AGING AFFECTS THE DRUG METABOLISM SYSTEMS OF RAT LIVER, KIDNEY, COLON AND LUNG IN A DIFFERENTIAL FASHION

JUNQI SUN† and HENRY W. STROBEL‡

Department of Biochemistry and Molecular Biology, The University of Texas Medical School at Houston, P.O. Box 20708, Houston, Texas

Abstract—Microsomes prepared from the liver, lungs, colon and kidney cortex of Sprague Dawley rats of ages 2, 4, 10, 24 and 78 weeks were assessed for hydroxylation activity with the substrate benzo[α]pyrene. Liver microsomal activity declined after reaching a peak of activity at 10 weeks. The hydroxylation of benzo[α]pyrene by colon, kidney and lung microsomes, however, either remained the same or decreased only slightly. During the age range examined inducibility of hydroxylation activity by β-naphthoflavone decreased with age in liver but actually increased with age in the extrahepatic tissues. Although phenobarbital did not elicit any increases in liver, kidney or lung, it increased substantially benzo[α]pyrene hydroxylation activity in colon microsomes of 78 week old rats. Total cytochrome P-450 content was induced at all age groups in all tissues by β-naphthoflavone and in all tissues except lung by phenobarbital. Induction of cytochrome P-450 in kidney by phenobarbital was only observed in 24 and 78 week old rats. These data suggest an increased role for extrahepatic activation of benzo[α]pyrene with aging. In contrast to total content of cytochrome P-450, the β-naphthoflavone inducible amount of Form 5 which has a high turnover number for benzo[α]pyrene, declined by 55% in liver between 2 weeks and 78 weeks while it increases dramatically in all extrahepatic tissues (from 80 to 138%).

Key Words: drug metabolism system, cytochrome P-450, aging and maturation, benzo[α]pyrene hydroxylation, extrahepatic drug metabolism, carcinogen activation

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INTRODUCTION

IT IS well recognized that aging affects the metabolism of drugs as well as many other physiological activities and functions. This recognition has led to the use of reduced doses of drugs in older patients in an effort to minimize overdose or other side effects (Gillette, 1979; Schmucker, 1979). Although age-related changes in drug metabolism may be the

Correspondence to: H.W. Strobel, Dept. of Biochemistry and Molecular Biology, The University of Texas Medical School at Houston, P.O. Box 20708, Houston, TX 77225.

†Present address: Department of Pharmacology, Beijing Second Medical School, Beijing, People's Republic of China.

‡Recipient of Research Career Development Award 1-K04-CA00311.

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result of many discrete effects, alteration in the activities of the drug metabolism system enzymes have been demonstrated directly in the liver of various species. Kato and coworkers (Kato *et al.*, 1961; Kato and Takanaka, 1968; Kato *et al.*, 1967) demonstrated that the activity of drug metabolizing enzymes was higher in young rats than in older rats and further showed that induction by phenobarbital of hepatic drug metabolism activities was higher in young rats than in older rats. However, aging appears not to decrease either the drug metabolism activity or the phenobarbital inducibility of mouse liver (Kato *et al.*, 1970), suggesting species differences in responses to aging. Kato and coworkers also showed a decline with age in the cytochrome P-450 specific content and cytochrome P-450 reductase activity in rat liver but not in mouse liver (Kato *et al.*, 1961; Kato and Takanaka, 1968; Kato *et al.*, 1970; Kato *et al.* 1967) Baird *et al.* (1970) have suggested that the age-related decline in zoxazolamine hydroxylation in rat liver may be the result of decreases with aging in cytochrome P-450 reductase activity.

Alterations of drug metabolism activity in specific tissues have also been shown to markedly affect the pharmacological effects of various xenobiotics or endogeneous compounds within tissues. Wood and coworkers Wood et al., 1976; 1979 for instance, have shown dramatic changes in the fate of benzo[α]pyrene and benzo[e]pyrene due to alterations of the amount and distribution of enzyme components present in reaction mixtures. These authors also showed that manipulation of drug metabolism system components dramatically affects the ultimate mutagenicity of benzo[α]pyrene and benzo[ϵ]pyrene by favoring the formation of particular metabolites.

These reports raise the question of whether the effects of aging on hepatic drug metabolism system activities also reflect aging effects in other tissues. This present paper compares the effects of aging on drug metabolism activities, inducibility, and distribution of one form of cytochrome P-450 in extrahepatic tissues with the effects of aging on these parameters in liver in male rats ranging in age from 2 weeks to 1.5 years.

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MATERIALS AND METHODS

Experimental Methods

Animals. Male Sprague Dawley rats from 2 weeks to 1.5 years in age were obtained shortly before use from the colony of aging Sprague Dawley rats which were maintained for the National Institute on Aging by the Charles River Laboratories. The rats were housed in wire-bottom cages and maintained on Purina lab chow and water *ad libitum*. At sacrifice the age and body weight of the rats used in these experiments were 0.5 month, 30 g; 1 month, 100 g; 2½ months, 203 g; 6 months, 551 g; and 18 months, 623 g, respectively. The eighteen-month-old rats were retired breeders. Phenobarbital pretreated rats received two intraperitoneal injections of 75 mg of phenobarbital sodium/kg body weight on the third day before sacrifice and two daily injections of phenobarbital sodium (75 mg/kg body weight) plus hydrocortisone-21-sodium succinate (50 mg/kg body weight) in saline on the second and first days preceding sacrifice. Hydrocortisone was used in these experiments because it enhances phenobarbital induction (Lu *et al.*, 1969). β-naphthoflavone-pretreated rats receive intraperitoneally 80 mg β-naphthoflavone/kg body weight in corn oil once daily for three days. Control rats received 1.0 ml corn oil or saline. Animals were fasted for the last 24 h preceding sacrifice with the exception of the two-week-old rats.

These acute treatments did not cause any significant change in body weight, sleeping patterns or food consumption.

Preparation of microsomes. All animals were dispatched by decapitation. The liver, lungs, colon and kidneys of each rat were removed and microsomes prepared by procedures optimized for each tissue as described below. The colons were immediately isolated from the end of the caecum to the end of the rectum, flushed with ice-cold "colon medium" (20 mM Tris-hydrochloride, pH 7.7, containing 0.14 M KCl, 20 mM EDTA, 0.25 M sucrose, 1.0 mM dithiothreitol, and 0.25 mM phenylmethylsulfonyl fluoride). The colons were everted and the mucosal cells were scraped with a microscope slide into fresh colon medium. The colon cells were homogenized in a glass-Teflon homogenizer and centrifuged twice at 500 × g for 5 min. The supernatant fraction was centrifuged at 9,000 × g for 10 min. The 9000 × g supernatant fraction was then centrifuged at 105,000 × g for 60 min in an ultracentrifuge to pellet the microsomal fraction. The microsomal pellet was resuspended in 0.25 M sucrose containing 20 mM EDTA (pH 7.7) and stored at −70°C under nitrogen gas.

Immediately after removal of the colon, the liver, lungs, and kidney of each rat was perfused *in situ* with 30–50 ml of ice cold 1.14% KCl solution containing 10 mM EDTA (pH 7.5) through the right and left heart chambers. The whole lungs were removed, dissected free of larger bronchi and other tissues, washed twice in ice-cold 0.15 M KCl blotted dry, and subsequently weighed. The entire lungs were minced finely with scissors in 10 ml of ice-cold 0.05 M Tris-chloride buffer containing 1.14% KCl/0.1M potassium phosphate buffer, pH 7.5, washed two times with same buffer, and homogenized using a glass-Teflon homogenizer. The homogenate, adjusted to a 20% suspension (w/v), was spun at 9,000 × g for 15 min; the supernatant was centrifuged twice at 105,000 × g for 90 min. The microsomal pellet was suspended in 0.25 M sucrose-10 mM EDTA solution (pH 7.5) and stored at −70°C under nitrogen gas. The livers were washed twice and homogenized in a Waring Blendor in 4 volumes of 1.14% KCl containing 10 mM EDTA (pH 7.5) to which phenylmethylsulfonyl fluoride was added to a final concentration of 0.25 mM immediately prior to cell disruption. Microsomes were collected by differential centrifugation as described above.

The kidneys were removed and immediately cooled in ice-cold 0.25M sucrose containing 10 mM EDTA (pH 7.5). The medulla was removed and the cortex was homogenized in 3 mM Tris-Cl buffer, pH 7.5 with a Waring Blender (PMSF was added to a final concentration of 0.25mM) for 1 min. The homogenate was spun at 10,000 × g for 10 min, and the supernatant was ultracentrifuged at 105,000 × g for 60 min; the microsomal fraction was washed once with 0.15 M Tris-Cl buffer, pH 8.0 and recentrifuged at 105,000 × g for 30 min. The pellets were suspended in 0.25 M sucrose containing 10 mM EDTA (ph 7.5) and stored at −70°C under nitrogen gas.

Assays. The hydroxylation of benzo[α]pyrene was measured at 30°C in 1.0 ml reaction mixtures incubated for 10 min containing 100 μmol potassium phosphate buffer (pH 7.7), 80 nmol benzo[α]pyrene and 0.15 μmol NADPH by fluorimetric estimation of the 3-hydroxy benzo[α]pyrene and other products according to the method of Nebert and Gelboin (1968) using an excitation wavelength of 386 nm and an emission wavelength of 515 nm. This assay provides a simple direct measure of the rate of hydroxylation of ben-

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zo[α]pyrene and was chosen for its overall utility despite of the fact that it does not assess differences in metabolite profile. Cytochrome P-450 specific content was measured from the dithionite reduced carbon monoxide difference spectrum according to the method of Omura and Sato (1964) using an extinction coefficient of 91 cm^{-1} mM^{-1} or according to the method of Miyake *et al.* (1974). NADPH-cytochrome P-450 reductase activity was measured by a modification (Dignam and Strobel, 1977) of the method of Phillips and Langdon (1962) at 30°C in 1.0 ml reaction mixtures containing 300 μmol potassium phosphate buffer (pH 7.7) as the rate of reduction of cytochrome c, using an extinction coefficient of 21 cm^{-1} mM^{-1} at 550 H nM (Williams and Kamin, 1962). Radial immunodiffusion was performed by a modification (Strobel and Lau, 1982) of the method of Thomas *et al.* (1979) using rabbit antibody to purified rat cytochrome P-450 Form 5. Each value is the average of triplicate determination of each of 6–9 experimental groups. Microsomes were prepared by pooling livers, kidneys, lungs or colons from 4 to 6 experimental animals either treated or untreated. Protein concentration was assayed by the method of Lowry *et al.* (1951).

Materials. Horse heart cytochrome c was purchased from Sigma Chemical Co., St. Louis, MO.; NADPH, from Boehringer Mannheim. Benzo[α]pyrene was obtained from Eastman Co., Rochester, N.Y. All other chemicals were reagent grade or better.

RESULTS

Effects of aging on drug metabolism activity

In order to compare the effects of aging on drug metabolism activity in liver with extra hepatic tissues, the rate of benzo[α]pyrene hydroxylation activity was measured in microsomes isolated from various organs of Sprague-Dawley rats at the ages of 2, 4, 10, 24 and 78 weeks. Benzo[α]pyrene was chosen as a substrate because its hydroxylation can be assessed by a sensitive flourimetric assay; and, while it is metabolized to some extent by most forms of cytochrome P-450, its primary first pass metabolism is catalyzed by Form 5 (Form C) which is specifically induced by β-naphthoflavone (Lau and Strobel, 1982) or 3-methylcholanthrene (Ryan *et al.*, 1979). Thus by use of benzo[α]pyrene as substrate we could not only test aging effects on the general activity of the drug metabolism system in various tissues, but we could also correlate these effects on activity with effects on a specific form of cytochrome P-450.

Benzo[α]pyrene hydroxylation activities in the liver, colon, kidneys and lungs of control, phenobarbital and β-naphthoflavone-induced rats shown as a function of age in Fig. 1. Benzo[α]pyrene hydroxylation activity is detectable without induction in all tissues in each age group. At all except the earliest ages the specific activity in liver is approximately 10 times higher than that of the other tissues. In control animals the peak activity is attained at 4 weeks in the extrahepatic tissues but not until 10 weeks in liver. With age the rates of benzo[α]pyrene hydroxylation activity in control animals drop to a constant level of about 50% of peak activity in the liver and extrahepatic tissues. β-Naphthoflavone has been shown to induce benzo[α]pyrene hydroxylation activity in liver and colon microsomes of young animals (Fang and Strobel, 1978; Saito and Strobel, 1981). In all tissues at all age groups benzo[α]pyrene hydroxylation activity is inducible by β-naphthoflavone (Fig. 1). In liver, the highest levels of benzo[α]pyrene hydroxylation were seen after

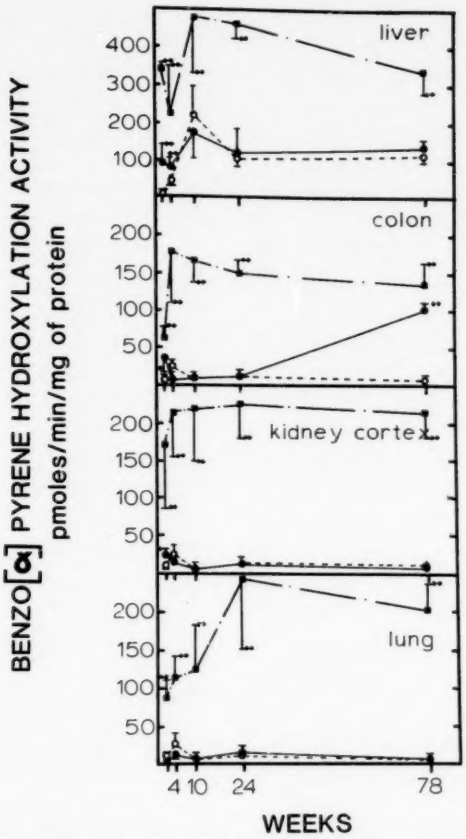

FIG. 1. Benzo[α]pyrene hydroxylation specific activity in liver, colon, kidney cortex and lung microsomes as a function of age. Microsomes were prepared and assayed for benzo[α]pyrene hydroxylation activity measured as described in the Methods section. Rats of ages 2,4,10,24 and 78 weeks were pretreated with phenobarbital (75 mg/Kg) plus hydrocortisone (50 mg/Kg) or β-naphthoflavone (80 mg/Kg) as described. Values for the control rats are designated by (○-○-○), phenobarbital/hydrocortisone pretreated rats by (●-●-●) and β-naphthoflavone-pretreated rats by (■-■-■). |'s represent the standard deviation of each point. ◆ indicates point significantly different from the control ($P = 0.05$, Student t test); ◆◆ significantly different from the control ($P = 0.01$, Student t test).

β-naphthoflavone treatment of 10-week-old rats. The total activity after treatment with β-naphthoflavone declines by 33%, however, when 78 week old rats are used. The activity at 78 weeks was significantly lower than that at 24 weeks ($P \geq 0.05$) but the activity levels at 24 weeks are not significantly different from the activity at 10 weeks. Inducibility of benzo[α]pyrene hydroxylation therefore also declines with aging in agreement with the results of others showing a decreased inducibility of drug metabolism with age (Baird *et al.*, 1970; Kato *et al.*, 1970; Kato *et al.* 1967). Interestingly, the 4 week old group appears to show a marked decrease both in activity and inducibility with β-naphthoflavone. This age group, however, when examined for N- or O- demethylation activities show both high

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activity and high inducibility by phenobarbital (data not presented). Schmucker and Wang (1980) report that Fischer 344 rats of this age group, in contrast, exhibit rates of ethylmorphine N-demethylation significantly lower than those measured in either 3- or 6-month-old animals.

In the colon benzo[α]pyrene hydroxylation activity shows dramatic inducibility by β-naphthoflavone at all age groups.

Although induction appears to decline by 18% from peak levels at 4 weeks to 78 weeks, the changes are not statistically significant. Inducibility changes from 12, 7, 18, 17, and 16-fold at 2, 4, 10, 24 and 78 weeks respectively. In the kidney, peak induction is achieved at 4 weeks and no drop in induced activity is observed with aging. Inducibility rises with aging from 10-fold at 4 weeks to 26-fold at 78 weeks. The lung exhibits peak induced activity at 24 weeks and does not decline significantly at 78 weeks. Inducibility is 9-, 4-, 24-, 20-, and 50-fold at 2, 4, 10, 24 and 78 weeks respectively, again increasing with aging.

The effects of phenobarbital on benzo[α]pyrene hydroxylation activity as a function of age are also shown in Fig. 1 (closed circles). Phenobarbital does not significantly change benzo[α]pyrene hydroxylation activity from control values in any tissue at any time period except for the colon in the oldest age group. Phenobarbital pretreatment induced colonic benzo[α]pyrene hydroxylation activity 12-fold over control levels in 78-week-old rats. This induction is significant both when compared to control values ($P \leq 0.01$) and when compared to the activity in colon microsomes from 24-week-old rats ($P \leq 0.05$). This induction was also correlated (see below) with an increase in the colon of a specific cytochrome P-450 with a high turnover number for benzo[α]pyrene (Lau and Strobel, 1982).

Thus as judged by alterations in the level of benzo[α]pyrene hydroxylation activity and by the changes in inductive responses to phenobarbital and β-naphthoflavone treatment, the aging process differentially affects hepatic and extrahepatic tissues.

Effects of age on cytochrome P-450 and cytochrome P-450 reductase

Although differential responses of hydroxylation activity in the various tissues with aging can be shown, the molecular basis needs elucidation. How are the component enzymes of the cytochrome P-450 drug metabolism system affected by aging? Metabolism by the P-450 system of a given substrate may be limited by total specific content of cytochromes P-450, by a specific isozyme of P-450, or by the rate at which electrons are supplied to the cytochrome by NADPH-cytochrome P-450 reductase.

Cytochrome P-450 is detectable in all tissues in all age groups of the uninduced rats as shown in Fig. 2. The patterns of variation in the total specific content of cytochrome P-450 are similar to the patterns of benzo[α]pyrene hydroxylation in several tissues. Cytochrome P-450 is inducible by β-naphthoflavone in all tissues at all ages. Phenobarbital pretreatment also induces cytochrome P-450 specific content at all age levels in all tissues except lung, where phenobarbital has no inductive effect, and in the kidney cortex where it does not induce cytochrome P-450 levels in young rats. Schmucker and Wang (1980) have shown that the induction of cytochrome P-450 by phenobarbital is more reduced in 27-month-old rats than in 16-month-old or 1-month-old rats. Our data are in good agreement with the observations of these workers.

Pretreatment with β-naphthoflavone increases the benzo[α]pyrene turnover number (hydroxylation rate/specific content of cytochrome P-450) over untreated animals in all

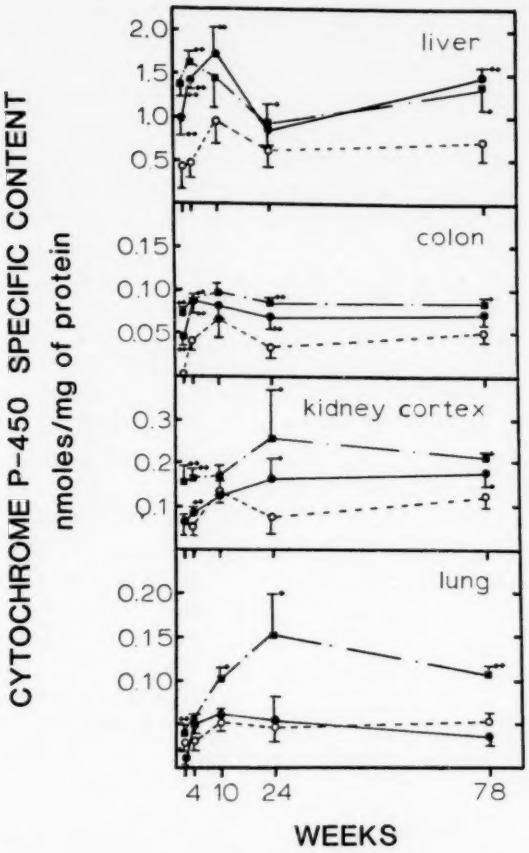

FIG. 2. Cytochrome P-450 specific content in liver, colon, kidney cortex and lung microsomes as a function of age. Procedural details are identical to those described in the legend in Fig. 1.

tissues at all age levels. Phenobarbital, however, decreased the turnover number for benzo[α]pyrene versus controls in all tissues except lung. The lung shows no significant change in either benzo[α]pyrene hydroxylation activity or cytochrome P-450 specific content after phenobarbital pretreatment. These results indicate that phenobarbital induces forms of cytochrome P-450 with low activity towards benzo[α]pyrene whereas β-naphthoflavone induces forms with high activity.

A single isozyme of cytochrome P-450 Form 5, can account for most hydroxylation of benzo[α]pyrene, due to its high turnover number. This is especially true upon its induction by β-naphthoflavone (Lau and Strobel, 1982). Can this single form account for the correlation between the age-related increase in activity and specific content seen upon induction with β-naphthoflavone and the lack of changes with induction by phenobarbital? We have shown by resolution and characterization studies that cytochrome P-450 isozyme Form 5 isolated from young and older rats are the same, as judged by molecular weight determination on SDS gels, CO difference spectrum maxima and Ouchterlony double dif-

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fusion immunochemical assay (Sun *et al.*, 1986). We approached this question by using an immunoquantitation technique (Strobel and Lau, 1982; Thomas *et al.*, 1989) with antibodies prepared to cytochrome P-450 Form 5. As shown in Table 1, the amount of Form 5 in liver microsomes from 78-week-old rats is about half of what is present in 2-week-old rats. In contrast the content of cytochrome P-450 Form 5 in kidney cortex, lung and colon microsomes from the older rats is substantially higher (from 80% for colon to 133% for kidney cortex) than the content in younger rats. Thus aging has a pronounced differential effect on induction of Form 5 in liver vs. extrahepatic tissues and this effect parallels the observed changes in the levels of benzo[α]pyrene hydroxylation activity in the various tissues. This result provides evidence that the molecular basis of the activity changes in the tissues may be the level of this single isozyme, Form 5.

Variations in cytochrome c reductase activity with aging are shown in Fig. 3. Reductase activity in untreated liver reaches peak activity at 10 weeks and declines to between 25 and 50% of peak activity thereafter. This observation is consistent with the trend of age related decline of activity described by the elegant studies of Schmucker and Wang (1983) using solubilized and purified preparations of cytochrome c reductase from young and aging male Fisher 344 rats. Phenobarbital significantly induces reductase activity in the liver at all age levels. This observation constitutes another similarity between the behavior of aging Sprague Dawley rats and the Fisher 344 rats used by Schmucker and Wang (1983). In the extrahepatic tissues, phenobarbital induction of reductase is significant only at 4 weeks in the colon, at 24 weeks in the kidney and 4 and 24 weeks in the lung. β-naphthoflavone induction of reductase activity is significant in the liver at 4, 24 and 78 weeks and in the kidney and lungs at 4 and 24 weeks. The correlation between the activity of the reductase and hydroxylation of benzo[α]pyrene is poorer than the correlation of hydroxylation activity and concentration of Form 5. This is in accord with other evidence that Form 5 of cytochrome P-450 may be a primary factor accounting for the changes in hydroxylation activity with induction.

DISCUSSION

The data reported in this study substantiate the results of others (Baird *et al.*, 1970; Birnbaum and Baird, 1978; Birnbaum and Baird, 1978a; Kato *et al.*, 1961; Kato and

TABLE 1. IMMUNOQUANTITATION OF CYTOCHROME P-450 FORM 5 BY RADIAL IMMUNODIFFUSION IN MICROSOMES OF FOUR TISSUES OF β-NAPHTHOFLAVONE PRETREATED RATS AT AGE 2 WEEKS AND 78 WEEKS

	Cytochrome P-450 Percent[a]	
	2 weeks	*78 weeks*
Liver	100	45
Colon	100	180
Kidney Cortex	100	238
Lung	100	190

[a]For each tissue the content of Form 5 at 2 weeks is set at 100%.

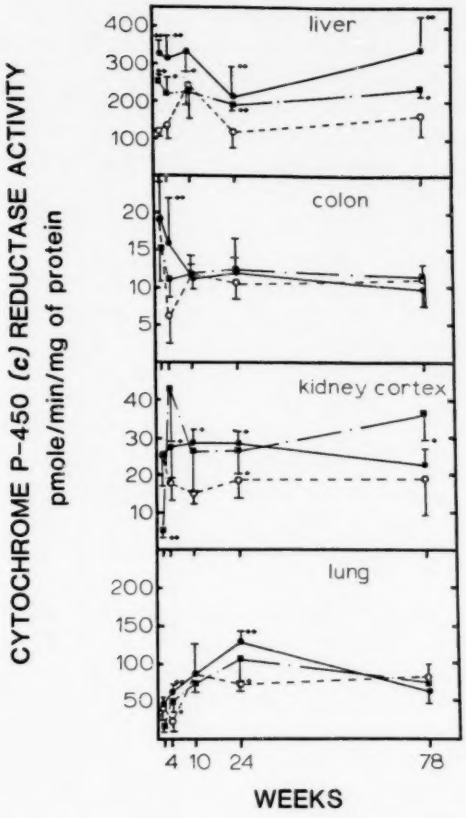

FIG. 3. Cytochrome c reductase specific activity in liver, colon, kidney cortex and lung microsomes as a function of age. Procedural details are identical to those described in the legend to Fig. 1.

Takanaka, 1960; Kato *et al.*, 1970; Kato *et al.*, 1967) who have shown that activity and inducibility of the hepatic drug metabolism generally decline with age. However, this study further shows that extrahepatic tissues show little decline in hydroxylation activity with aging when compared to the liver and in some cases show a relative increase in inducibility of hydroxylation activity with aging. These data raise the possibility that extrahepatic tissues may become increasingly important with age in the metabolism of benzo[α]pyrene and perhaps of other drugs as well.

Substrate specificity is a property of the cytochrome P-450 component of the drug metabolism system (Lu *et al.*, 1972). Variation in microsomal cytochrome P-450 composition (e.g. from tissue to tissue) can markedly change the profile of metabolites derived from a particular substrate. In addition, recent reports have shown marked gender and age related shifts in specific liver microsomal cytochrome P-450 isozymes (Kamataki, 1985; Kitagawa *et al.*, 1965; Morgan *et al.*, 1985). Thus it is possible for a change in the amount of a specific form of cytochrome P-450 present in a tissue to result in a shift of the metabolite profile of a substrate to produce more carcinogenic metabolites. Wood *et al.*

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(1976, 1979) have demonstrated that altering the forms of cytochrome P-450 present in liver by pretreatment with various inducers can increase the amount of mutagenic or carcinogenic metabolites produced from a specific precarcinogen. Aging, which as we have shown brings about changes in the drug metabolism system, metabolic activity of liver and other tissue, may play a role in carcinogenesis. Baird and Birnbaum (1979) for instance, have shown that the hepatic 9000 × g supernatant fraction and microsome fraction from senescent rats and mice produce more mutagenic products from several procarcinogens than fractions from younger animals. Alterations in drug metabolism activities in the various tissues may be related to other changes which combine to modify total drug metabolism. Stohs *et al.* (1980) have reported decreases in reduced glutathione levels in various tissues of mice with age. Age related alterations in hepatic mixed function oxidases such as reported for mice (Al-Turk *et al.*, 1981) may be enhanced by the putative lowered conjugation ability (Stohs *et al.*, 1980) perhaps contributing to the limited hepatic drug handling capability seen in old age (Gillette, 1979; Schmucker, 1979).

The age-dependent changes in total cytochrome P-450 specific content and the inducibility of total cytochrome P-450 specific specific content in each tissue are in relative agreement with age-dependent alterations in benzo[α]pyrene hydroxylation activities in the respective tissues (see Figs. 1 and 2). These changes in activity and total cytochrome P-450 content reflect the differential effect of aging on liver in comparison to the three extrahepatic tissues.

Comparison of the age related changes in the content of a specific cytochrome P-450 form (Form 5), moreover, allows a further description of the effects of aging on drug metabolism and possible consequences. The data presented in Table 1 suggest that the quantitative distribution of cytochromes P-450 may be affected differentially by the aging process. Specifically, Form 5 significantly increases with aging in the extrahepatic tissues while it decreases in the liver. Marked changes in the content of individual forms of cytochrome P-450 by inducers with only small effects on total specific content have been observed by others as well (Domin *et al.*, 1983). Thus, a relatively constant response to inducers of total cytochrome P-450 may mask a differential response in the component isozymes.

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While these variations occur with aging it must be pointed out that in the time range we have utilized the rats are subject to all the hormonal effects brought on by maturation. (e.g. shifts in testosterone levels circulating in the blood). Therefore these observations must be interpreted cautiously. Nonetheless differential effects of aging on the liver versus the extra hepatic tissues appear even over maturation induced changes. The dramatic induction of colonic, but not hepatic, benzo[α]pyrene hydroxylation by phenobarbital treatment of 18-month-old rats demonstrates this age related differential response to inducers.

The aging process might also affect the properties or method of response to induction of the component enzymes of the drug metabolism system. For instance, Schmucker and Wang (1983, 1983a) have reported age-related changes in the physical and chemical properties of hepatic cytochrome P-450 reductase. In a similar vein our data point out a dramatic age-related change in the response of colonic cytochrome P-450 to induction. In tissues other than the colon at all ages tested, benzo[α]pyrene hydroxylation is induced by pretreatment with β-naphthoflavone but not with phenobarbital. In marked contrast to these results, phenobarbital pretreatment of 78 week old rats elicited a marked induction in benzo[α]pyrene hydroxylation as well as the expected increase in benzphetamine hydroxylation. Moreover, the induction by phenobarbital of benzo[α]pyrene

hydroxylation in 78 week old rats was equal to the induction elicited with β-naphthoflavone, the known inducer of benzo[α]pyrene hydroxylation. Whether this represents a loss in fidelity at the level of response to inducers or the production of a variant form of cytochrome P-450 is not known. This observation is consistent, however, with a rise in the dimethylhydrazine activation reported in human colon with aging (Newaz *et al.*, 1983).

The differential effects of aging on liver in contrast to the extrahepatic tissues suggest the speculation that more drugs may reach extrahepatic tissue in older individuals than in younger individuals. If true, then the distribution of drug metabolizing enzymes and activities in peripheral tissues may play an increasingly significant role in the metabolic fate of drugs whether carcinogenic or chemotherapeutic. Thus, assessment at the molecular level of the effects of aging on the drug and carcinogen metabolism systems of extrahepatic tissues is increasingly necessary to develop an adequate understanding of the effects of aging on disease processes.

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AN ELECTRON MICROSCOPIC EXAMINATION OF AGE-RELATED CHANGES IN THE RAT KIDNEY: THE INFLUENCE OF DIET

M. HAYASHIDA[1], B.P. YU[2], E.J. MASORO[2], K. IWASAKI[2,3] and T. IKEDA[3]

[1]Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan;
[2]Department of Physiology, University of Texas Health Science Center, San Antonio, Texas 78284;
[3]Department of Pathology, Nagasaki University School of Medicine, Nagasaki, Japan

Key Words: electron microscopy, rat kidney, diet, age, chronic nephropathy

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INTRODUCTION

CHRONIC NEPHROPATHY is a common age-related disease process in rats (Coleman *et al.*, 1977; Anver *et al.*, 1982; Gray *et al.*, 1982). Its characteristics in some respects are similar to atherosclerosis in humans in that it progresses with increasing age, and every rat has lesions, although of varying severity. The functional expression of the nephropathy includes elevated blood urea nitrogen, elevated serum creatinine, parathyroid hyperplasia, osteodystrophy and metastatic calcifications. The pathophysiologic findings occur late in life, but many rats die without their occurrence (Maeda *et al.*, 1985).

It is well known that nutrition modulates age-related progression of chronic nephropathy (Saxton and Kimball, 1941; Berg and Simms, 1960; Bras and Ross, 1964; Yu *et al.*, 1982). In a study with male Fischer 344 rats, just completed in our laboratory, food restriction and protein restriction during different portions of the life span have been found to variably modify the progression of chronic nephropathy (Maeda *et al.*, 1985). The analysis of these kidneys by light microscopy has been published (Maeda *et al.*, 1985). The present paper is a report of further examination of these kidneys by transmission electron microscopy. Although there have been a few reports in which electron microscopy has been used to study the chronic nephropathy of rats (Gray *et al.*, 1974; Hirokawa, 1975; Kreisburg and Kamorsky, 1978; Bolton and Sturgill, 1981), nothing has been done in regard to the nutritional modulation of these lesions in aging rats. One report using scanning electron microscopy has been published on nutritional effects on morphologic changes with age in the kidneys of mice (Johnson and Barrows, 1980).

Correspondence to: Dr. Byung Pal Yu, Department of Physiology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284.
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MATERIALS AND METHODS

Rat Maintenance and Dietary Program

Male Fischer 344 rats (specific pathogen free, 4 weeks of age weanlings) were received in a single shipment from the Portage, MI plant of the Charles River Laboratories. To maintain the specific pathogen-free condition, on arrival, the rats were transferred into a barrier facility where they were singly housed. The procedures for their maintenance including the monitoring for infectious disease, the dietary protocols, the food intake data, and the body weight data were described previously (Yu *et al.*, 1985).

In brief, all rat groups were fed Diet A (caloric composition of 21% protein, 57% carbohydrate, 22% fat) *ad libitum* until 6 weeks of age. Group 1 continued to receive Diet A *ad libitum* until death or sacrifice. Group 2 was restricted to 60% of the food intake on Group 1 from 6 weeks of age on; the diet during the food restriction was Diet B which is similar to Diet A except for fortification with vitamins, Ca, P and Na to provide the rats in Group 2 the same daily intake of these substances as the rats in Group 1. Group 3 was fed Diet B at 60% of the food intake of Group 1 from 6 weeks to 6 months of age and thereafter fed Diet A *ad* libitum. Group 4 was fed Diet A *ad libitum* until 6 months of age and thereafter given Diet B at 60% of the food intake of Group 1. Group 5 was fed Diet C (caloric composition of 12.6% protein, 65.4% carbohydrate, 22% fat) *ad libitum* from 6 months of age on. The rats in Group 5 had the same caloric intake as the rats in Group 1 and the same protein intake as the rats in Group 2. The dietary protocol is summarized in Fig. 1.

Preparation of Kidneys for Electron Microscopy

Rats were anesthetized with methoxyflurane in a chamber which is equilibrated with oxygen as described previously and killed by exsanguination (Masoro *et al.*, 1983). Both kidneys were removed, sliced into 1–2 mm cubes, and immersion-fixed in 0.1M cacodylate buffered 2% glutaraldehyde. The kidney blocks were washed in 0.1M cacodylate buffer, then post-fixed in 1% OsO_4 0.1 M cacodylate buffer at neutral pH for 1 h at room temperature and embloc stained with aqueous uranyl acetate. They were dehydrated in an

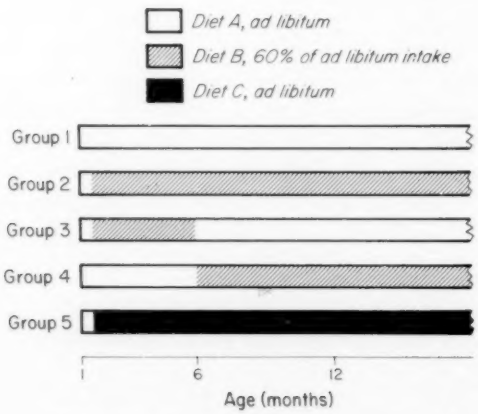

FIG. 1. Graphic summary of dietary protocol.

elevated series of ethanol solutions and then embedded in Spurr's resin (Spur, 1969). Seven or eight blocks were prepared from each rat. For the purpose of identifying and orienting the glomeruli and tubules, all blocks were cut into 0.6–1 μm thick sections and were stained with toluidine blue. Glomeruli were included in more than 90% of sections. The sections without glomeruli were not used for observation.

From proper blocks, ultra thin sections were prepared and stained with uranyl acetate and lead citrate. These specimens were examined with both Philip 301 and Joel 100CX electron microscopes. Glomeruli and proximal tubules were examined and 10–15 photographs were taken for each rat. Glomerular basement membrane, epithelial cell, foot processes, mesangium, Bowman's capsule, tubular basement membrane and tubular epithelial cells were photographed at medium (3300×) and high magnification (5000× or 8000×). Thickness of basement membrane was measured for 15 regions randomly selected regardless of the grade of lesions in proximal tubules or glomeruli from each rat on the electron micrographs. The effects of age and diet on glomerular epithelial cell, foot processes, mesangium, and the tubular epithelial cells were analyzed utilizing the high magnification photographs. Grading of pathological changes was analyzed by two pathologists and estimated by comparing Group 1 with other groups.

Statistical Analysis

The data were analyzed by analysis of variance. Tests of differences between dietary groups and differences between time trends in the dietary groups were made using linear contrasts.

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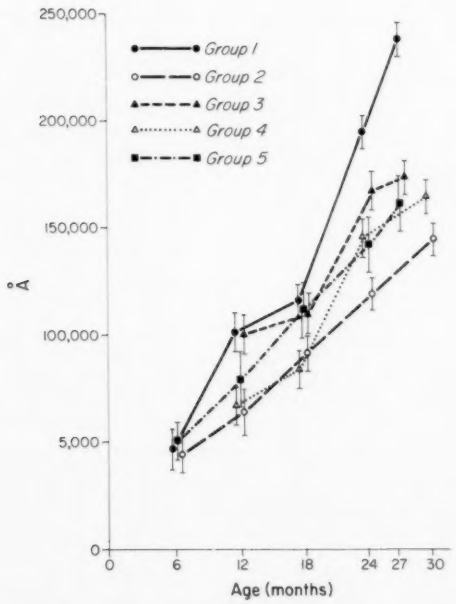

FIG. 2. Age-related changes in thickness of glomerular basement membrane.

RESULTS

Glomerular Basement Membrane

As reported previously (Maeda *et al.*, 1985), light microscopy revealed age changes in the glomerular basement membrane that were modified by diet. Such changes were further explored using the transmission electron microscope. Data on the thickness of the glomerular basement membrane of rats of each of the dietary groups are presented in Fig. 2. In all dietary groups, the thickness increased with increasing age ($P < 0.005$). The extent of increase was greatest for the rats of Group 1 and least for those of Group 2 ($P < 0.005$).

To assess the effects of age and diet on the ultrastructure of the glomerular basement membrane, the changes that occur in the ultrastructure of basement membrane with age are classified in Table 1. Examples of most of these lesions can be found in the micrographs of Fig. 3. The frequency of occurrence of these lesions in relation to age and dietary regimen is presented in Table 2. The following is a summary of the major findings for each of the rat groups:

Group 1: Focal thickening with distinct three layers (lesion Ia) was observed in all rats 6 to 18 months of age but not in 24- and 27-month-old rats. The lesion of focal thickening without distinct three layers (lesion Ib) was observed increasingly with age and by 24 months of age all rats showed this lesion. Scattered deposits of droplets or vesicles (lesion IIa) were only seen occasionally. Massive deposits of a variety of droplets or vesicles (lesion IIb) were not observed in glomerular basement membranes. Mottled areas in the thickened basement membrane (lesion III) were not observed until 24 months of age when all rats had the lesion. Multilamellar structure (lesion IV) was not observed until 24 months of age and at no age did all rats have this lesion. Protrusion of lamina interna or externa (lesion Va) was rarely seen and none of the collagen bundle was seen in the basement membrane.

Groups 3 and 5: Although the pattern of glomerular lesions in the Group 3 and 5 rats was similar to that of the Group 1 rats, there were some differences. Many of the 24- and 27-month-old rats in these groups had the lesion of focal thickening with distinct three layers in the basement membrane. Also, in Group 5 there was not an increase in frequency of the lesion with deposits or vesicles at 24 and 27 months of age.

Groups 2 and 4: The lesions in Group 2 rats were similar to that of Group 1 rats but were delayed in regard to age of appearance. Group 4 rats also showed a delay in ap-

TABLE 1. CLASSIFICATION OF ULTRASTRUCTURE LESIONS IN BASEMENT MEMBRANES

Lesion Ia:	Focal thickening of lamina densa with distinct three layers
Lesion Ib:	Focal thickening of lamina densa without distinct three layers
Lesion IIa:	In addition to focal or diffuse thickening, a few small and scattered deposits of droplets or vesicles are seen.
Lesion IIb:	In addition to thickening, massive deposits of a variety of droplets or vesicles are seen.
Lesion III:	In addition to lesion IIb, a mottled area is present.
Lesion IV:	In addition to lesion III, multilaminated structures are present.
Lesion Va:	Mild protrusion of lamina interna or externa with flocculent or fibrillary materials
Lesion Vb:	Moderate protrusion similar to lesion Va, with collagen bundles

TABLE 2. FREQUENCY OF CATEGORIES OF GLOMERULAR BASEMENT MEMBRANE LESIONS*

		Age of Rats					
		6 mos.	*12 mos.*	*18 mos.*	*24 mos.*	*27 mos.*	*30 mos.*
Group 1	Ia	4/4	4/4	5/5	0/5	0/5	
	Ib	0/4	2/4	4/5	5/5	5/5	
	IIa	0/4	1/4	0/5	2/5	2/5	
	IIb	0/4	0/4	0/5	0/5	0/5	
	III	0/4	0/4	0/5	5/5	5/5	
	IV	0/4	0/4	0/5	2/5	3/5	
	Va	1/4	0/4	0/5	0/5	0/5	
	Vb	0/4	0/4	0/5	0/5	0/5	
Group 2	Ia	4/4	3/3	4/4	2/5		1/5
	Ib	0/4	0/3	0/4	1/5		5/5
	IIa	0/4	1/3	0/4	0/5		0/5
	IIb	0/4	0/3	0/4	0/5		0/5
	III	0/4	0/3	0/4	1/5		0/5
	IV	0/4	0/3	0/4	0/5		1/5
	Va	0/4	0/3	0/4	0/5		0/5
	Vb	0/4	0/3	0/4	0/5		0/5
Group 3	Ia		4/4	3/3	4/4	3/5	
	Ib		2/4	1/3	4/4	5/5	
	IIa		0/4	0/3	1/4	4/5	
	IIb		0/4	0/3	0/4	0/5	
	III		0/4	0/3	1/4	5/5	
	IV		0/4	0/3	1/4	0/5	
	Va		0/4	0/3	0/4	0/5	
	Vb		0/4	0/3	0/4	0/5	
Group 4	Ia		4/4	4/4	5/5		1/5
	Ib		3/4	4/4	5/5		5/5
	IIa		3/4	0/4	2/5		5/5
	IIb		0/4	0/4	0/5		0/5
	III		0/4	0/4	0/5		3/5
	IV		0/4	0/4	0/5		1/5
	Va		0/4	0/4	0/5		0/5
	Vb		0/4	0/4	0/5		0/5
Group 5	Ia	4/4	4/4	4/4	3/3	1/2	
	Ib	1/4	2/4	3/4	3/3	2/2	
	IIa	1/4	1/4	0/4	0/3	0/2	
	IIb	0/4	0/4	0/4	0/3	0/2	
	III	0/4	0/4	0/4	2/3	2/2	
	IV	0/4	0/4	0/4	1/3	0/2	
	Va	0/4	0/4	0/4	0/3	0/2	
	Vb	0/4	0/4	0/4	0/3	0/2	

*X/Y: X refers to the number of rats with lesion and Y to the number of rats examined.

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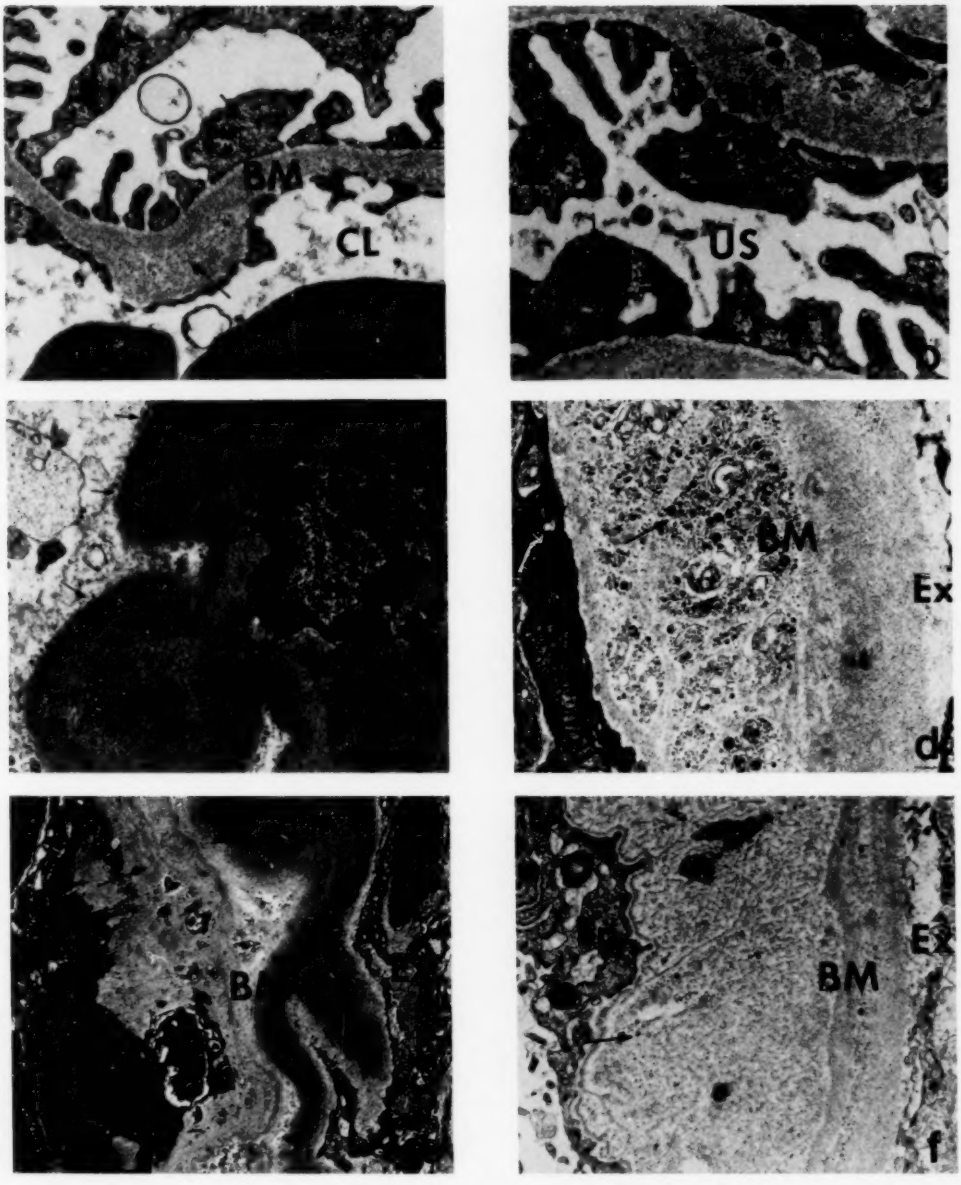

FIG. 3. Micrographs illustrating the lesions classified in Table 1: a. Lesion Ia. in a glomerulus (×22100). This lesion is observed in younger rats in all dietary groups. Focal thickening of basement membrane (long arrow) and focal fusion of foot process are also noted (short arrow). b. Lesion IIa. in a glomerulus (×27600). This lesion is most frequently observed in aged rats in Groups 1, 3, and 5. A few deposit of droplets or vesicles (long arrow) and focal and zonal fusions of foot process and epithelial body (short arrow) are also noted. c. Lesion III. in a glomerulus (×11000). Massive deposits of droplets or vesicles are noted (long arrows). Disappearance of foot process is noted (short arrows). d. Lesion III. in a tubular epithelium

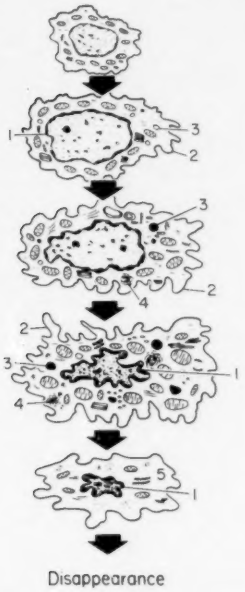

FIG. 4. Age-related changes of glomerular epithelial cells. Note an increased cytoplasmic and nuclear volumes, and subsequent atrophy and disappearance. Arrows denote the age-related progression and the numbers refer the following specific morphologic entities: 1. nuclear indentation; 2. microvillous formation; 3. vesicles; 4. myelin figure; 5. degeneration and atrophy of cell.

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pearance of glomerular basement membrane lesions compared to Group 1 rats but less delay than noted for Group 2 rats.

Glomerular Epithelial Cells

The morphologic changes in glomerular epithelial cells that can occur with age are diagrammed in Fig. 4. Age changes in morphology and the effects of diet are reported in Table 3.

Group 1: Nuclear indentation, microvillous formation and vesicles were observed in rats of all ages. The size and number of vesicles increased with age as did myelin figures. Cell degeneration and atrophy became increasingly common with age.

Groups 3 and 5: Group 3 had less myelin figures and Group 5 much less than Group 1 even at advanced ages. Age-associated cell degeneration and atrophy were less marked in rats from Groups 3 and 5 than in those of Group 1. There was little difference between rats in Groups 1, 3 and 5 in regard to nuclear indentation, microvillous formation, and size and number of vesicles.

(×14200) Mottled areas is noted (arrow). e. Lesion IV. in a tubular epithelium (×4200). Multilaminated structure is noted (arrows). f. Lesion Vb. in a tubular epithelium (×11700). Moderate protrusion in basement membrane is noted (arrow). (Abbreviations: CL-capillary lumen, US: urinary space: BM-basement membrane; EP-tubular epithelium; Ex-extracellular space; F-foot process.)

TABLE 3. GLOMERULAR EPITHELIAL CELL CHANGES WITH AGE AND DIET

	Age of Rats					
	6 mos.	*12 mos.*	*18 mos.*	*24 mos.*	*27 mos.*	*30 mos.*
Group 1						
nuclear indentation[a]	+ +	+ + +	+ + +	+ +	+ +	
microvillous formation[b]	+ +	+ + +	+ + +	+ +	+	
number of vesicles[c]	+	+ + +	+ + +	+ + +	+ + +	
size of vesicles[c]	S	M	M	L	L	
myelin figures[d]	0	+ +	+ +	+ + +	+ + +	
cell degeneration[e]	0	+ +	+ + +	+ + +	+ + +	
cell atrophy[f]	0	0	0	+	+ + +	
Group 2						
nuclear indentation[a]	+ +	+ +	+	+ +		+ +
microvillous formation[b]	+ +	+ +	+ + +	+ + +		+ + +
number of vesicles[c]	+	+	+	+ + +		+ + +
size of vesicles[c]	S	S	S	S		S
myelin figures[d]	0	0	0	0		+
cell degeneration[e]	0	0	0	+		+
cell atrophy[f]	0	0	0	0		+
Group 3						
nuclear indentation[a]	+ +	+ + +	+ +	+ +	+ +	
microvillous formation[b]	+ +	+ + +	+ + +	+ +	+ +	
number of vesicles[c]	+	+ + +	+ + +	+ + +	+ + +	
size of vesicles[c]	S	M	M	M	M	
myelin figures[d]	0	0	+	+ + +	+ + +	
cell degeneration[e]	0	0	+ +	+ +	+ +	
cell atrophy[f]	0	0	0	+	+ +	
Group 4						
nuclear indentation[a]	+ +	+ + +	+ +	+ +		+ +
microvillous formation[b]	+ +	+ + +	+ + +	+ +		+ +
number of vesicles[c]	+	+ + +	+ + +	+ + +		+ + +
size of vesicles[c]	S	M	M	M		M
myelin figures[d]	0	0	+	+ +		+ +
cell degeneration[e]	0	0	+ +	+ +		+ +
cell atrophy[f]	0	0	0	+		+
Group 5						
nuclear indentation[a]	+ +	+ + +	+ +	+ +	+ +	
microvillous formation[b]	+ +	+ + +	+ + +	+ +	+ +	
number of vesicles[c]	+	+ + +	+ + +	+ + +	+ + +	
size of vesicles[c]	S	M	M	L	L	
myelin figures[d]	0	+	+	+	+	
cell degeneration[e]	0	+	+	+	+ +	
cell atrophy[f]	0	0	0	+	+	

[a]Nuclear indentation (numbers)—0: about 20% of the nuclei (normal); +: about 30%; + +: about 50%; + + +: more than 60%.

[b]Microvillous formation—+: less frequent than normal; + +; frequent (normal); + + +: more frequent than normal.

[c]Number of vesicles (in 10 μm^2)—+: less than 10; + +: 11–15; + + +: more than 15. Size of vesicles—S: less than 0.2μm in diameter; M: 0.2–0.5μm in diameter; L: more than 0.5μm in diameter.

[d]Myelin figures—0: none or less than one per cell (normal); +: 1–2 per cell in number; + +: 3–5 per cell in number; + + +: more than 5 per cell in number.

[e]Cell degeneration (a cell showing the occurrence of cytolysome and osimiophilic change of cytoplasm is referred to as a degenerated cell)—+: less than 20% of the cells in number; + +: 20–40%; + + +: more than 40%.

[f]Cell atrophy (a cell showing a decrease to less than 2/3 in diameter is referred to as astrophy)—0: less than 5% of the cells; +: 5–10%; + +: 10–20%; + + +: more than 20%.

Groups 2 and 4: Most of the age changes such as the increase in sizes and number of vesicles, accumulation of myelin figures, degeneration and atrophy of cells were less and occurred at much later ages with Group 2 rats. A similar modulation of these age changes was seen with Group 4 rats but its extent was not as great as for Group 2.

The changes in foot processes of glomerular epithelial cell that occur with age are shown in Fig. 5 and their occurrence in the various dietary groups is recorded in Table 4. In Group 1, 3 and 5 rats, the variation in the size and shape of the foot processes increased with age until 18 months of age after which it decreased. This age-related increase in variation was also seen in Group 2 and 4 rats, but was delayed with the peak occurring at 24 months of age in the Group 4 rats and at 30 months in the Group 2 rats. There was a pro-

TABLE 4. CHANGES IN FOOT PROCESS WITH AGE AND DIET*

	Age of Rats					
	6 mos.	*12 mos.*	*18 mos.*	*24 mos.*	*27 mos.*	*30 mos.*
Group 1						
variation of size	+	+ +	+ + +	+ +	+	
focal fusion	+	+ +	+ +	+ + +	+ + +	
zonal fusion	0	+	+ +	+ + +	+ + +	
extensive fusion	0	+	+ +	+ + +	+ + +	
atrophy	0	+	+ +	+ + +	+ + +	
Group 2						
variation of size	+	+	+	+ +		+ + +
focal fusion	+	+	+	+ +		+ + +
zonal fusion	0	0	0	+		+
extensive fusion	0	0	0	+		
atrophy	0	0	0	+		+
Group 3						
variation of size	+	+ +	+ + +	+ +	+ +	
focal fusion	+	+ +	+ +	+ + +	+ + +	
zonal fusion	0	0	+	+ +	+ +	
extensive fusion	0	+	+	+ +	+ +	
atrophy	0	+	+	+ +	+ + +	
Group 4						
variation of size	+	+ +	+ +	+ + +		+ +
focal fusion	+	+ +	+ +	+ + +		+ + +
zonal fusion	0	0	+	+		+ +
extensive fusion	0	0	+	+		+ +
atrophy	0	0	+	+ +		+ +
Group 5						
variation of size	+	+ +	+ + +	+ +	+ +	
focal fusion	+	+ +	+ +	+ +	+ + +	
zonal fusion	0	0	+	+ +	+ +	
extensive fusion	0	0	0	+	+ +	
atrophy	0	0	0	+	+	

*0 refers to no occurrence; + refers to occurrence; + + refers to frequent occurrence; + + + refers to very frequent occurrence.

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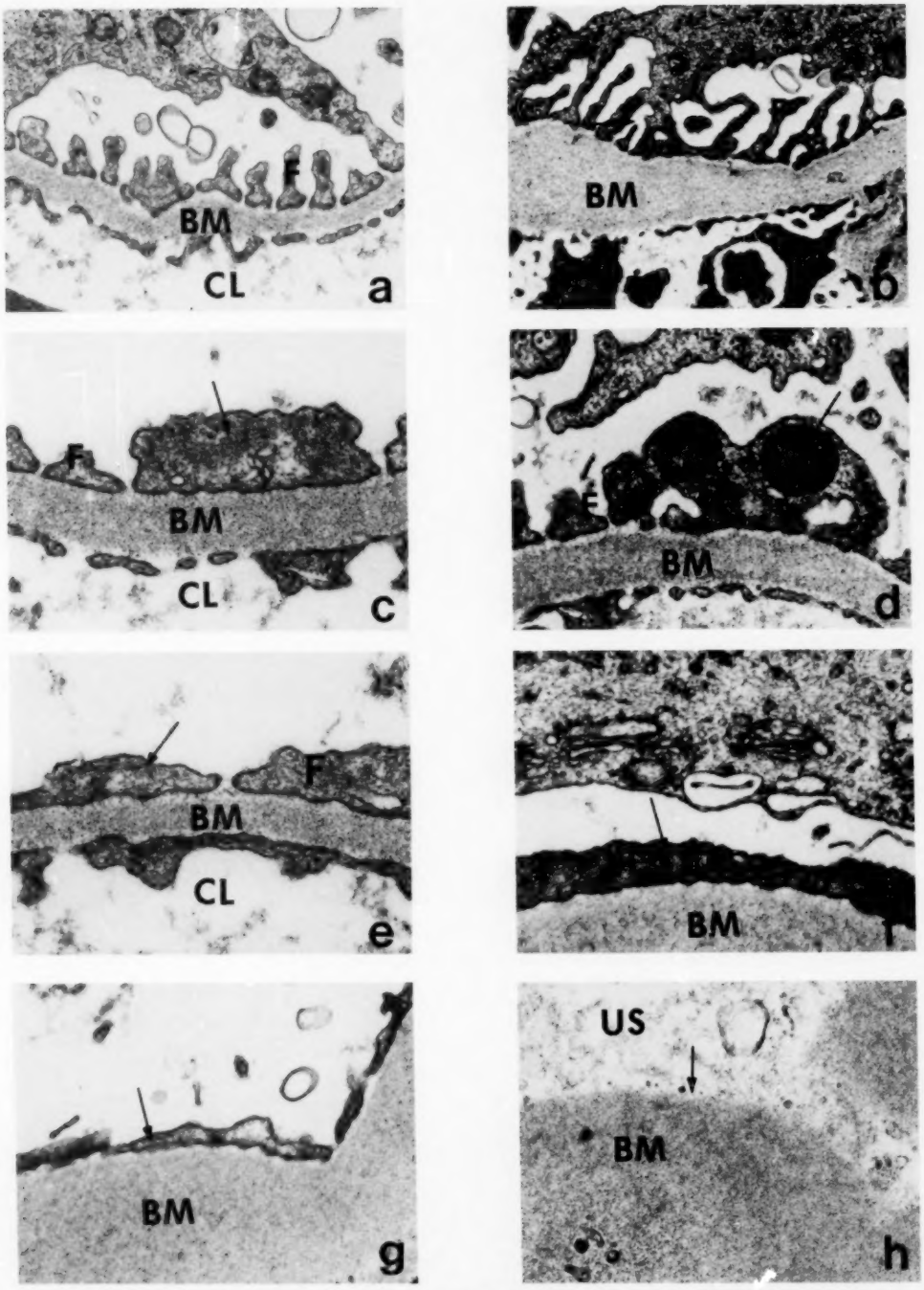

FIG. 5. Micrographs illustrating the changes in foot processes that occur with age: a. Normal foot process (×23600); b. villous formation, sometimes connected with a strand of cytoplasmic process (arrow, ×14600); c. focal and scattered fusions of foot processes (arrow,

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TABLE 5. CHANGES IN MESANGIUM WITH AGE AND DIET*

	Age of Rats					
	6 mos.	*12 mos.*	*18 mos.*	*24 mos.*	*27 mos.*	*30 mos.*
Group 1						
matrix size	+	+	+ +	+ + +	+ + +	
cell number	+ +	+ + +	+ + +	+ +	+ +	
fusion of mesangium	+	+	+ + +	+ + +	+ + +	
Group 2						
matrix size	+	+	+ +	+ +		+ +
cell number	+ +	+ +	+ +	+ +		+ + +
fusion of mesangium	+	+	+ +	+ +		+ + +
Group 3						
matrix size	+	+	+ +	+ + +	+ + +	
cell number	+ +	+ +	+ +	+ + +	+ +	
fusion of mesangium	+	+	+ +	+ + +	+ + +	
Group 4						
matrix size	+	+	+ +	+ +		+ + +
cell number	+ +	+ +	+ +	+ + +		+ + +
fusion of mesangium	+	+	+ +	+ +		+ + +
Group 5						
matrix size	+	+	+ +	+ +	+ +	
cell number	+ +	+ +	+ + +	+ + +	+ + +	
fusion of mesangium	+	+	+ + +	+ + +	+ + +	

*0 refers to no occurrence of fusion or all glomeruli with normal structure; + refers to some occurrence of fusion or to a few glomeruli with greater than normal matrix size or cell number; + + refers to the frequent occurrence of fusion or to many glomeruli with greater than normal matrix size or cell number; + + + refers to the very frequent occurrence of fusion or to most glomeruli with greater than normal matrix size or cell number.

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gressive increase with age in focal fusion in all groups with the rate of progression being the slowest in Group 2 rats. Zone fusion also increased with increasing age in all groups with the rate of increase being greatest in Group 1 rats and least Group 2 rats. Atrophy and disappearance of the foot processes increased with increasing age in all groups, occurring to the greatest extent in Group 1 rats and least in Group 2 and 5 rats.

Mesangium

The age changes that occur in the mesangium are shown in Fig. 6 and their occurrence in the rats of the various dietary groups is reported in Table 5. There were age-related increases in matrix size, cell number, fusion of mesangium and subsequent obliteration

×46000); d. In addition to change 3, epithelial body (infolded condensed material) is present (arrow, ×29000); e. segmental flattening and retraction of fused foot processes (arrow, ×46000); f. zonal flattening and retraction of fused foot processes (extensive fusion) (arrow, ×25000); g. atrophy of fused foot processes (arrow, ×24200); h. disappearance of foot process (arrow, ×24000). (Abbreviations: BM-basement membrane; CL-capillary lumen; F-foot process; US-urinary space.)

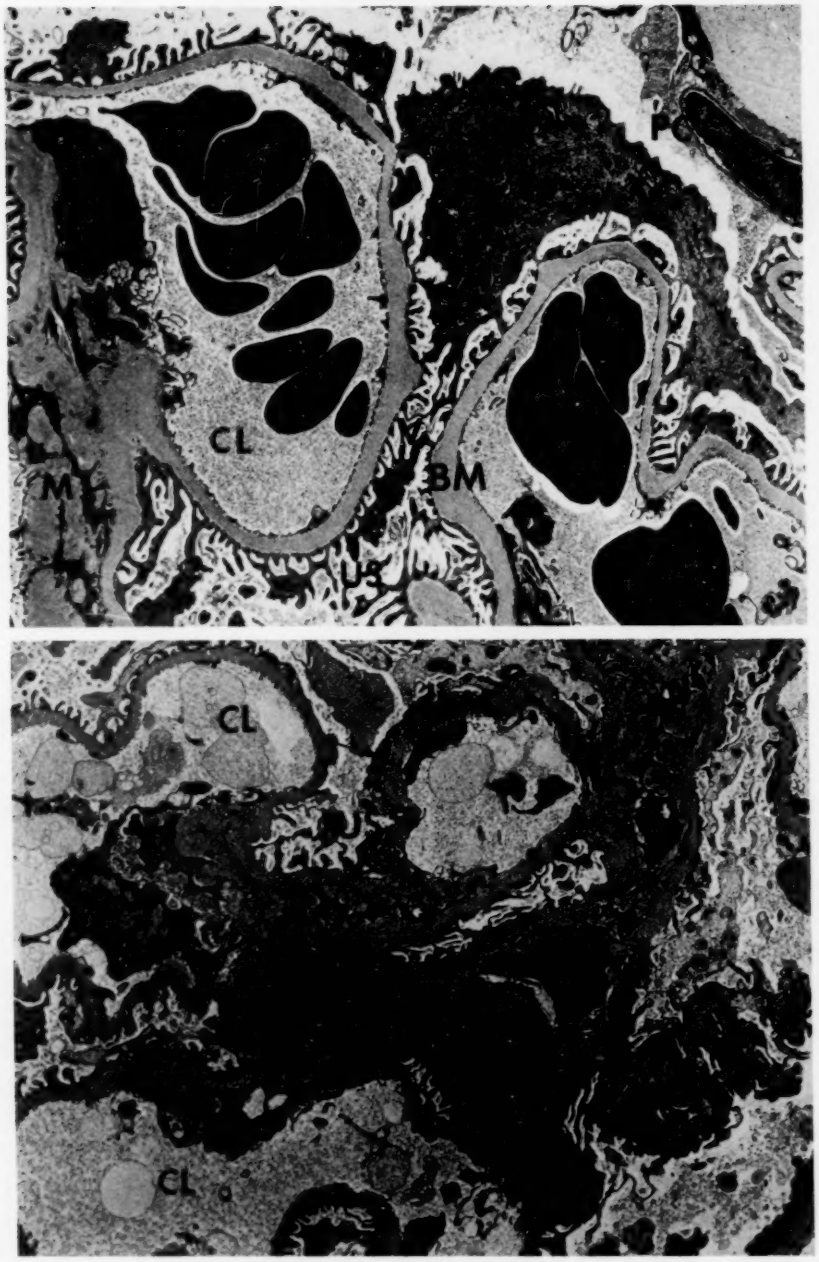
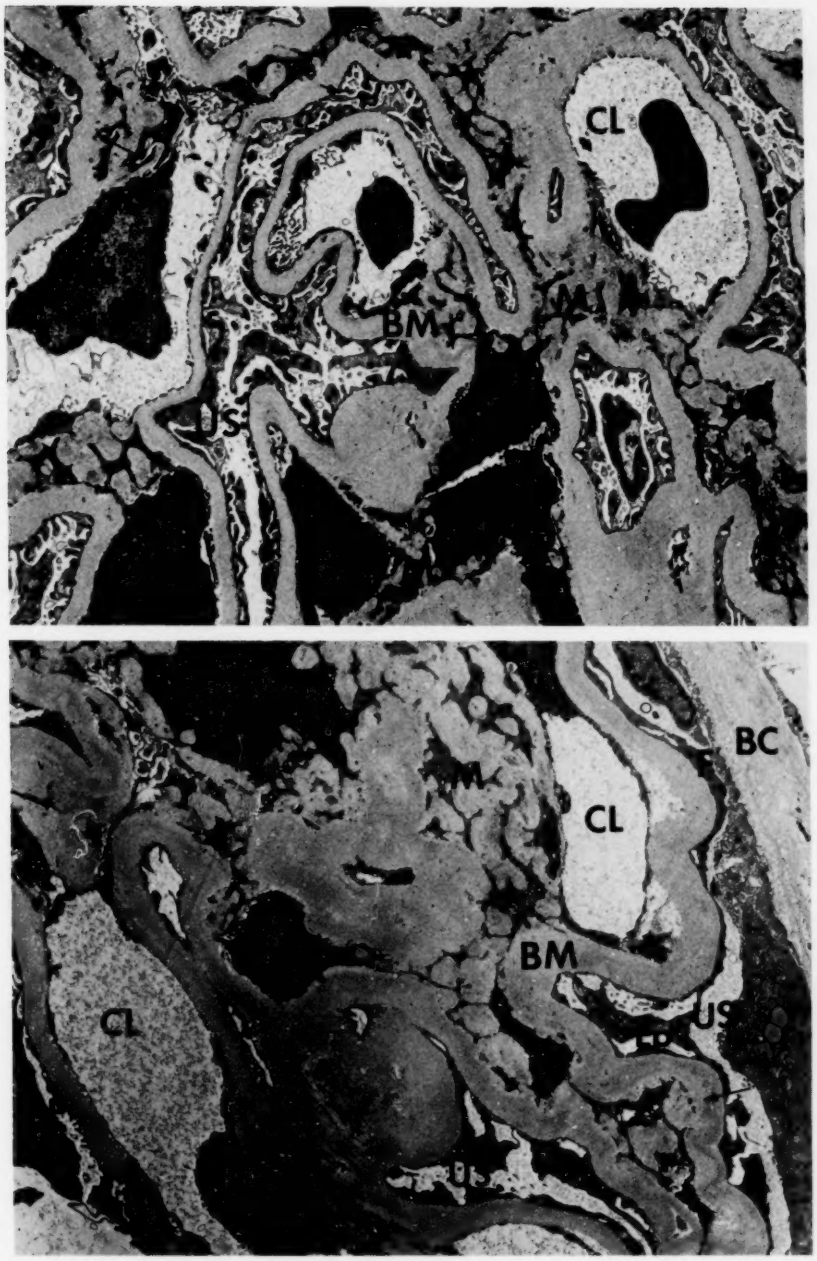


FIG. 6. Age-related changes in mesangium: The micrographs in Fig. 6a–6d show a gradual increase of mesangium with interlacing basement membrane-like material. In severly affected glomeruli this material is accumulated in a patchwork or nodular pattern in mesangium. Fusion of mesangium with subsequent obliteration of urinary space and frequent protrusion of mesangium into capillary lumen are noted. (Abbreviations: BC-Bowman's capsule; US-



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urinary space; Ep-glomerular epithelial cell; CL-capillary lumen; M-mesangium; PC-parietal epithelial cell of Bowman's capsule; F-foot process; V-villous formation): a. Micrograph of a glomerulus of a 12-month old Group 1 rat (×7000). Note focal and diffuse thickening of basement membrane, increase of mesangium, protrusion of mesangium into capillary lumen, and villous formation and connection of foot process. b. Micrograph of a glomerulus of a

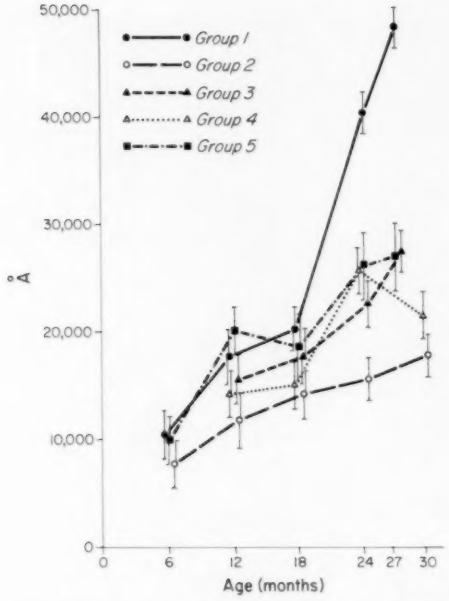

FIG. 7. Age-related changes in thickness of tubular basement membrane. Note similar findings to those observed in the glomerular basement membrane.

of urinary space in all groups. The progression in these parameters was less in Group 5 than for the other groups. Int he rats of Groups 1 and 3 the cell number decreased at advanced ages.

Tubular Basement Membrane

Changes in the thickness of the renal tubular basement membrane in relation to age and diet are presented in Fig. 7. The thickness increased with age in all groups ($P < 0.005$) and the increase was greatest for Group 1 rats ($P < 0.005$).

The classification of the pathologic lesions of the tubular basement membrane is the same as that presented for the glomerular basement membrane. The frequency of their occurrence is presented in Table 6. In contrast to the glomerular basement membrane, Ia lesions were not seen in the tubular basement membrane. All rats irrespective of diet had Ib

18-month old Group 1 rat: Note the increase of mesangium; the prominent villous formations (short arrow); the adhesion of the foot process (long arrow, ×5000). c. Micrograph of a glomerulus of a 24-month old Group 1 rat: Note age-changes in glomerular epithelial cell in villous formation including vesicles, degeneration (short arrow) and increased thickness and irregularity of basement membrane (long arrow). Also note the increase mesangial matrix, and the varying sizes of the mesangial cells (×5900). d. Micrograph of a glomerulus of a 27-month old Group 1 rat: Note the of fusion and atrophy of foot processes. Note also the further increase in the thickness and irregularity of the glomerular basement membrane (long arrow), the increase in mesangial matrix, the increased adhesion of the mesangium and the degeneration of the mesangial cell. Urinary space was obliterated (short arrow, ×5500).

TABLE 6. TUBULAR BASEMENT MEMBRANE CHANGE WITH AGE AND DIET*

		Age of Rats					
		6 mos.	*12 mos.*	*18 mos.*	*24 mos.*	*27 mos.*	*30 mos.*
Group 1	Ia	0/4	0/4	0/5	0/5	0/5	
	Ib	4/4	4/4	5/5	5/5	5/5	
	IIa	2/4	0/4	1/5	0/5	0/5	
	IIb	0/4	2/4	1/5	4/5	5/5	
	III	0/4	0/4	1/5	3/5	3/5	
	IV	0/4	0/4	1/5	1/5	3/5	
	Va	0/4	1/4	2/5	0/5	0/5	
	Vb	0/4	0/4	1/5	1/5	4/5	
Group 2	Ia	0/4	0/3	0/4	0/5		0/5
	Ib	4/4	3/3	4/4	5/5		5/5
	IIa	3/4	0/3	0/4	0/5		0/5
	IIb	0/4	0/3	1/4	5/5		3/5
	III	0/4	0/3	0/4	0/5		1/5
	IV	0/4	0/3	0/4	0/5		1/5
	Va	1/4	0/3	2/4	2/5		2/5
	Vb	0/4	0/3	0/4	1/5		1/5
Group 3	Ia		0/4	0/3	0/4	0/5	
	Ib		4/4	3/3	4/4	5/5	
	IIa		4/4	2/3	1/4	0/5	
	IIb		2/4	2/3	3/4	5/5	
	III		0/4	0/3	1/4	4/5	
	IV		0/4	0/3	0/4	1/5	
	Va		1/4	1/3	1/4	3/5	
	Vb		0/4	0/3	0/4	1/5	
Group 4	Ia		0/4	0/4	0/5		0/5
	Ib		4/4	4/4	5/5		5/5
	IIa		2/4	1/4	2/5		3/5
	IIb		2/4	2/4	4/5		4/5
	III		0/4	0/4	1/5		2/5
	IV		0/4	0/4	0/5		0/5
	Va		0/4	1/4	2/5		0/5
	Vb		1/4	0/4	0/5		0/5
Group 5	Ia	0/4	0/4	0/4	0/3	0/2	
	Ib	4/4	4/4	4/4	3/3	2/2	
	IIa	3/4	4/4	2/4	3/3	0/2	
	IIb	1/4	1/4	1/4	1/3	1/2	
	III	0/4	1/4	1/4	3/3	1/2	
	IV	0/4	0/4	0/4	0/3	1/2	
	Va	1/4	1/4	1/4	0/3	0/2	
	Vb	0/4	0/4	1/4	1/3	1/2	

*X/Y: X refers to the number of rats with lesion and Y to the number of rats examined.

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lesions throughout adult life. In general IIa lesions tended to decrease with increasing age and were less common in Groups 1 and 2 than in the other groups. In contrast, IIb lesions increased with increasing age in Groups 1, 2, 3 and 4. The IIb lesion was less common in Group 5 rats than in the other groups. There was an age-related increase in III lesions; Group 2 rats were almost free of this lesion. The occurrence of IV lesions increased with age in Group 1 rats and did not commonly occur in the rats of the other groups. Although Va lesions were seen in many of the animals, there was no clear relationship with diet or age. In Group 1, Vb lesions were common and appeared to increase with increasing age; this lesion was much less common in the other groups.

Tubular Epithelial Cells

The changes in the renal tubular epithelial cell that occur with age are diagrammed in Fig. 8. In Group 1 rats, there was an age-related occurrence of the following: a narrowing of the tubular lumen; an increasing intercellular space (400 to 500 Å in young rats to 2 to 3 times the value in old rats); an increasing infolding of basal surface until middle age followed by fusion of the cell to the basement membrane; a progressive disappearance of the brush border; a disordering of mitochondrial arrangement together with a change in shape and an increase in size (due to a larger fraction of large mitochondria in old compared to young with the predominant form being small in rats of all ages); an accumulation of autosomes, residual bodies and lipofuscin-like secondary lysosomes. The narrow-

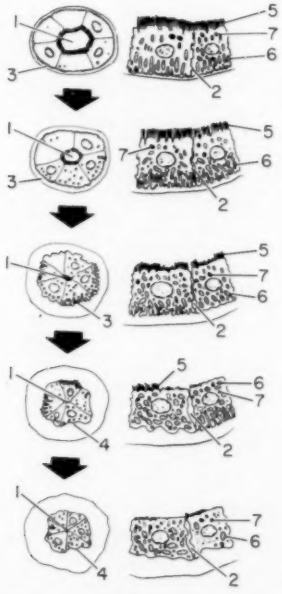

FIG. 8. Age-related changes of tubular epithelial cells. Arrows denote the age-related progression and the numbers refer to the following specific morphologic entities: 1. tubular lumen; 2. intercellular space; 3. basal surface; 4. fusion of basal surface of basement membrane; 5. brush border; 6. mitochondria; 7. autosome, residual body and lipofuscin-like secondary lysome. Note the narrowing of lumen, decrease of brush border, increase of autosomes, decrease of infolding of cytoplasm at basal surface and broadening of intercellular space with increasing age.

ing of the tubular lumen was estimated as follows: the luminal fractions of the total tubular diameter which ranged from a value of about one-third in most tubules of young animals to a vanishingly small value in the "occluded" tubules of *ad libitum* fed old rats. In Group 2 rats, the occurrence of these changes was markedly retarded. The cellular changes in Group 4 rats were similar to those in Group 2 rats but the age-related progression was somewhat greater. The changes in the tubular cells with age in Group 3 rats were similar to those described for Group 1 rats except for the fact that lipofuscin-like secondary lysosomes did not appear. The changes found with Group 5 rats were the same as those seen with Group 1 rats.

DISCUSSION

The glomerular basement membrane plays an essential role in renal function. Age-related changes in kidneys are characterized by the thickening of glomerular basement membrane. In the present study, the thickening of glomerular basement membrane increased with aging in almost a linear fashion. The mechanisms for this increase in glomerular basement membrane thickness are unknown. However, the collagen component of glomerular basement membrane originates from the visceral epithelium and turns over slowly being continuously removed by mesangial cells (Kurtz and Feldman, 1962; Walter, 1973). Thus, one possibility is that the accumulation of fibrillary material in older rats results from a decreased rate of breakdown of collagen and possibly other membrane components. Food restriction (i.e. Group 1 compared to Group 2) inhibited the age-related increase in thickness of basement membrane; protein restriction also inhibited this change (Group 1 compared to Group 5).

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The progressive thickening of the glomerular basement membrane with age is accompanied by subtle histochemical and electron microscopic alterations indicating a change in the chemical nature of the membrane. Moreover, other investigators have found that thickening of glomerular basement membrane involves changes in its composition (Daniels and Chu, 1975; Sato and Spiro, 1976; Taylor and Price, 1977). However, the relationship of these compositional changes to the pathologic changes classified in our study remains to be explored. In particular, how age and diet influence the composition of the basement membrane should be studied.

In addition to the changes in the basement membrane, many other age-related changes in the glomerulus were also found. Other investigators have reported that age-related changes of glomerular epithelial cells parallel the alterations in glomerular basement membrane thickness and structure (Bolton and Sturgill, 1981) and that compensatory changes, such as hypertrophy, fusion of foot processes, increased formation of hyaline or protein absorption droplets, and microvillar transformation of the cell surface, occur (Gray *et al.*, 1974). In our study, two types of age-related glomerular epithelial cell changes were observed. One type was the compensatory changes. The other type was the degenerative change in the glomerular epithelial cell such as cell degeneration and cell atrophy. Food restriction did not have a remarkable effect on compensatory changes but markedly retarded the degenerative changes. The various dietary regimens differed quantitative in their effectiveness with the dietary regimen of Group 2 being most effective.

The foot process changes with age such as villous formation, focal fusion and the presence of vesicles were only modestly influenced by food restriction. In contrast, other age-associated foot process changes such as diffuse fusion, atrophy and disappearance, which appear to be destructive in nature, were greatly modified by food restriction.

Proteinuria increases with age in rats (Coleman *et al.*, 1977), but the underlying mechanism is still unknown. The ultrastructural alterations noted in the present paper are probably relevant. Szarfman *et al.* (1982) have described two types of filtration barriers to protein in kidney, one is a size-dependent barrier regulated by arrangement of type IV collagen in glomerular basement membrane and the other is a charge-dependent barrier regulated by a negatively-charged proteoglycan. They postulate that the increase in thickness of the basement membrane reflects the change in the size-related barrier and that the other age changes in the glomerular basement membrane, e.g. that of the epithelial cell and foot process, reflect the changes in the charge-related barrier.

Enlargement and fusion of the mesangium are major age-related changes in renal morphology (Causer and Stilmant, 1975). These age-related mesangial changes are remarkably inhibited by food restriction (the dietary regimen of Group 2 being more effective than that of Group 4); restriction of protein but not calories (Group 5) was less effective.

The progressive thickening of the tubular basement membrane observed in the present study agrees with earlier observations in rats by Gray *et al.* (1974). The extent of increase in tubular basement membrane thickness in food-restricted rats (Groups 2) and protein-restricted rats (Group 5) is lower than in rats not so restricted (Group 1). These thickened tubular basement membranes show similar but not identical ultrastructural changes as the glomerular basement membranes. The renal tubules were sometimes filled with amorphous and moderately electron-dense materials with the tubular epithelium becoming flattened. It has been reported that there is an increase in dense bodies of the proximal tubule in old rats (Gray *et al.*, 1974), but this observation could not be confirmed by the present study. By morphometric analysis, Christensen *et al.* (1978) reported no significant change in the lysosomal system during aging. However, an increase with age in autosomes and lipofuscin-like secondary lysosomes was observed in the present study. These tubular changes are inhibited by food restriction but not by protein restriction.

In conclusion, many ultrastructural changes occur in the kidney of rats with increasing age and many of these changes are modulated by dietary manipulations. Thus, this study provides ultrastructural insight on the age changes in rat kidney that have previously been well characterized by light microscopic and functional measurements and further shows that the dietary manipulations retard age-related ultrastructural alterations.

SUMMARY

The changes with age in the ultrastructure of the kidneys were explored in *ad libitum* fed rats with restricted food intake started soon after weaning or started in young adult life or limited to early life and in rats restricted in protein but not caloric intake. Many ultrastructural changes occurred with age both in the glomeruli and the tubules. Food restriction started soon after weaning or in adult life modulated most of these age changes. By providing detailed information on basement membrane and tubular cell structure, these findings complement previous light microscopic and functional studies in regard to the effects of food restriction on progressive kidney disease in the rat. Food restriction limited to early life and protein restriction without caloric restriction were less effective in modulating these age changes in kidney ultrastructure than food restriction initiated at 6 weeks or 6 months of age and continued for the rest of the life span.

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AGE-ESTIMATIONS OF RATS BASED ON THE AVERAGE LATERAL DIFFUSION CONSTANT OF HEPATOCYTE MEMBRANE PROTEINS AS REVEALED BY FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING

I. ZS.-NAGY*, K. KITANI, M. OHTA, V. ZS.-NAGY* and K. IMAHORI

First Laboratory of Clinical Physiology, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho, Itabashi-ku, Tokyo-173, Japan

Abstract—A method has been developed recently for measuring the average lateral diffusion constant of the proteins (D) in the cell membrane of hepatocytes in liver smears by fluorescence recovery after photobleaching (FRAP). A peroxide-induced autofluorescence (PIAF) of the membrane proteins was used as a fluorescent label. It has been established that D displays a significant negative linear correlation with age. The present paper describes age-estimations carried out on 12 male Fischer 344 rats (7–29 months of age) in so-called "blind experiments": the operator knew only the sex of the rat, determined D from a small piece of the freshly removed liver, and estimated the age of the rat from the age-dependent regression line for D established previously on 16 other Fischer 344 male rats of various ages. There was a strong correlation of the estimated age with the actual one ($r = 0.92$), the slope of the regression line was 0.98 and its intercept differed from 0 by only 0.5 months. These results indicate that D may play a decisive role in the determination of membrane functions as predicted by the membrane hypothesis of aging.

Key Words: lateral diffusion of membrane proteins, fluorescence recovery after photobleaching, membrane hypothesis of aging, peroxide-induced autofluorescence, FRAP method for compact tissues.

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INTRODUCTION

THE LATERAL diffusion of membrane components has been a center of interest for the last decade. It can be measured by means of a special technique called fluorescence recovery after photobleaching (FRAP), or fluorescence photobleaching recovery (FPR). Several reviews (Axelrod *et al.*, 1976; Cherry 1979; Vaz *et al.*, 1982) treating the possibilities and limitations of this method are available. Although there are still some unresolved theoretical problems (Staffman and Delbrück, 1975; Kleinfeld, *et al.*, 1981), there is no doubt that

*Permanent address and correspondence to: ZS.-Nagy, F. Verzár International Laboratory for Experimental Gerontology (VILEG), Hungarian Section, University Medical School, H-4012, Debrecen, Hungary.
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the FRAP method can contribute to understanding the role of membrane components. It may also provide important data regarding the changes in membrane functions in various physiological and experimental processes like aging, pathologies, etc.

In our laboratory we have elaborated a FRAP method for use with compact tissue cells such as freshly removed liver (Zs.-Nagy *et al.*, 1984, 1986a). Apart from using compact tissue cells for this purpose, an interesting feature of our method was that we did not apply external fluorescent labels like lectins, antibodies, etc., since it is known that these components (e.g. the concanavalin-A-FITC label) cause a strong cross-linking of the membrane proteins, altering in this way both the diffusion constant and the mobile fraction of the membrane proteins (Schlessinger *et al.*, 1976; Lustyik *et al.*, 1986).

We previously reported that the smearing technique may be suitable for FRAP studies of compact tissues (Zs.-Nagy *et al.*, 1984; 1986a; 1986c). We also noted that hepatocytes in liver smears exposed to atmospheric oxygen pressure develop a yellowish-green autofluorescence localized on their membrane proteins and that this autofluorescence can be enhanced and stabilized by a mild H_2O_2 treatment (1 mM) of the liver smears (Zs.-Nagy *et al* , 1986a), therefore the appellation peroxide-induced autofluorescence (PIAF). The existence of PIAF offers a possibility for FRAP measurements without the use of any external fluorescent label.

When studying the nature of PIAF and the protein mobility in hepatocyte membranes of rats of different ages, we observed a highly significant negative linear correlation between the lateral diffusion constant of membrane proteins (*D*) and the age of Fischer 344 (Zs.-Nagy *et al.*, 1986a) and Wistar rats (Kitani *et al.*, 1986). It has also been established that male and female rats give regression lines of D with significantly different siopes (Zs.-Nagy *et al.*, 1986a). This high correlation suggested that on the basis of a previously established regression line of *D* it would be possible to perform age-estimations in so-called "blind" experiments. The main finding was that *D* is a suitable parameter for such purposes, i.e., *D* may be of great importance in the membrane functions.

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MATERIALS AND METHODS

Animals

Sixteen male Fischer 344 rats were used for these experiments in the age range of 7 to 29 months. The rats were killed by decapitation and bled for about 30 sec in a tail up position (except 1 case where there may have been a faulty desanguination). Pieces of the liver were excised within another half a minute, and given to the operator who knew only the sex of the animal (a "blind experiment"). In these experiments pathohistological analysis of liver samples from all animals was performed and considered in the final results.

Specimen preparation

Immediately after removal of the tissue from the body, the liver smears were prepared between two glass slides. Details of this method are described elsewhere (Zs.-Nagy *et al.*, 1986a, 1986c), therefore, we shall summarize only the main steps of this procedure.

Development of the autofluorescence

The liver smear was placed horizontally in a Petri dish and covered with 1 ml of Krebs-Henseleit bicarbonate Ringer solution of pH 7.4 (Dawson *et al.*, 1969) containing 1 mM

H_2O_2. In keeping with the technique described in our previous paper (Zs.-Nagy *et al.*, 1986a), the smear was incubated at 37°C for 10 min, washed 3 × 1 min in peroxide-free buffer and finally covered by the same buffer and sealed with melted paraffin.

PIAF is yellowish-green when excited by blue laser light of 476 nm and its intensity varies within the smear. The highest intensities of uniform distribution were present in the vicinity of air-bubbles remaining under the cover glass.

The FRAP measurement

The instrumental setup and computer handling of data was the same as described elsewhere (Zs.-Nagy *et al.*, 1986a). All measurements were carried out using an OLYMPUS FLPL objective (×40, NA = 0.75) giving a spot half diameter (W) at the intensity of Ie^{-2} in average $W = 1.5$ μm as measured by densitometry (Zs.-Nagy *et al.*, 1984, 1986a).

The design of the FRAP experiment is shown in Fig. 1. An observation period of 100 s was used during which the stability of the fluorescence was determined. After the observation period the program automatically performed the bleaching of the fluorescence by opening the shutter of the main laser beam for 100 ms, and then recording the recovery curve for another 100 s. The PIAF intensity was measured during each fourth second (Fig. 1).

The mathematical elaboration of the recovery curve was performed as described in detail elsewhere (Zs.-Nagy *et al.*, 1986a). Essential points of this procedure are the calculations of F(0), (the fluorescence intensity at zero postbleach time), and F(inf), (the maximum recovery) (Fig. 1). From the recovery curve one also obtains the characteristic diffusion time (τ) and the diffusion constant (D). In Fig. 1 *DD* indicates the average of D calculated by two independent methods as previously described (Zs.-Nagy *et al.*, 1986a).

After, making the measurements, the data obtained were checked for Gaussian distribution. This analysis was developed to exclude the consideration of cells which were in a state of early autolysis, as explained elsewhere (Zs.-Nagy *et al.*, 1986a). The individual average of D was then used to estimate the age of the rat from the age-dependent regression line of D previously established on 16 male Fischer 344 rats. The correlation between

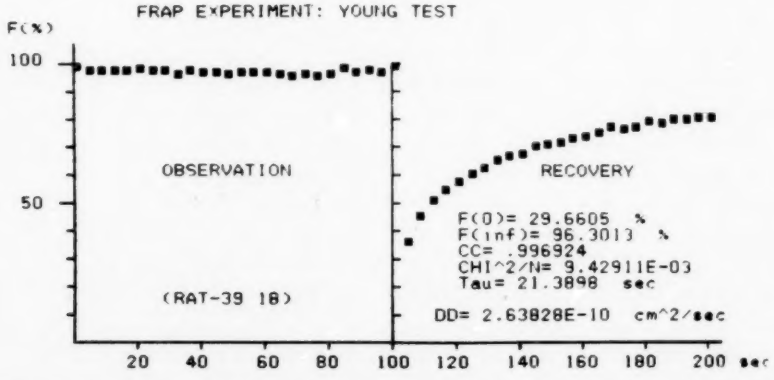

FIG. 1. A computer plot of a FRAP measurement of a young male rat. The initial intensity of PIAF is very stable (the "observation" period). A regular recovery curve was recorded between 100 and 200 s of the experiment. The figure shows the parameters calculated from the recovery curve (explained in the text).

the real and estimated ages of the rats was calculated by a computer program based on the best fit method of least squares (Snedecor and Cohran, 1980).

RESULTS

The electron microscopic morphology of the hepatocytes in the smear was determined during these experiments as described in our previous papers (Zs.-Nagy *et al.*, 1986a, 1986c). Transmission electron microscopy of perpendicular ultrathin sections of the liver smears showed that the majority of the hepatocytes maintained a clean cell membrane surface in the uppermost layer of the smear even after incubation with H_2O_2. Disrupted cells and some debris occurred occasionally, however, such cells were not common during the first 2–3 h. Furthermore, D values obtained from disrupted cells were excluded from the results by determining the Gaussian distribution as will be described below. Subsequently, we encountered more disrupted cells in the smears indicating that the autolysis of hepatocytes proceeds spontaneously. Autolysis also manifests itself as a time-dependent increase in the swelling of the intracellular organelles such as mitochondria, endoplasmic reticulum, etc. (Zs.-Nagy *et al.*, 1986c). Since autolysis proved to be age-dependent (Zs.-Nagy *et al.*, 1986a), and the age of the animals was unknown during the present experiments, the liver smears used for the FRAP measurements were at most 2–3 h old, i.e., a period suitable for old rats (Zs.- Nagy *et al.*, 1986a).

The pathohistological analysis revealed no alterations in the liver in 12 of 16 cases. These 12 animals were considered healthy and their data were used in our age-estimations. The others displayed some alterations which will be noted below.

First we will summarize the information regarding the Gaussian distribution of the values of D within each animal. As we described elsewhere (Zs.-Nagy *et al.*, 1986a), the histogram classes were chosen so that the hypothesis of normal distribution of the given sample would be accepted if $\chi^2 < 6$ (Snedecor and Cohran, 1980). In one animal no correction of the data was needed, since this figure was 1.13, however, in all other cases we had to correct our data, i.e. to omit cells in early autolysis with D values far out of the Gaussian distribution of the normal cells. The average value of χ^2 mounted to 4.25 after the corrections which consisted of omitting 31 cells from 376 (i.e. 8.2%). That is, we obtained 345 "good" measurements from 12 animals, or 28.7 (S.D. = ± 10.6) cells/animal on average.

The next step of our analysis was the comparison of the values of D from the "blind" experiments with those obtained from the nonblind measurements and performed identically. Fig. 2 shows the result of such a comparison. The first conclusion to be drawn is that addition of the data from the "blind" experiments did not change the original regression line, since all the parameters remained practically identical. The regression coefficient for the nonblind experiments was 0.98 (Zs.- Nagy *et al.*, 1986a), whereas for the "blind" ones it was 0.92 (Fig. 2). The common correlation coefficient of all 28 male rats is 0.96. The slope of the linear regression line remained also practically identical (Fig. 2). These results indicate that our method for the measurement of D is highly reliable and gives reproducible results.

One can also demonstrate the fit of the age-estimations by plotting the real age of the rats against the estimated one as shown in Fig. 3. Obviously, the linear regression line should have a slope of unity and its intercept should be zero. In our case the slope proved to be 0.98 and the intercept was -0.5 months, i.e. the estimated ages correspond quite well to the actual ones. The average standard error of the age-estimations expressed as the

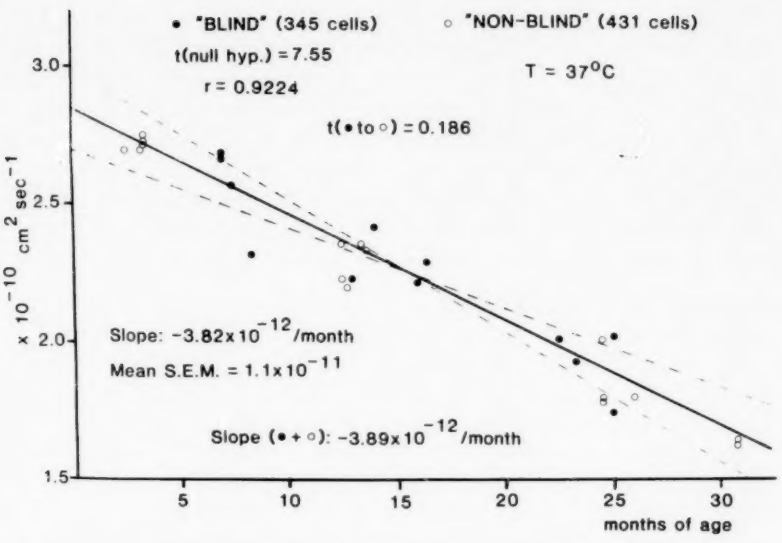

FIG. 2. Comparison of "blind" and nonblind FRAP measurements of the diffusion constant of membrane proteins (ordinate) as plotted against age (abscissa) in male Fischer 344 rats. Measurements performed at 37°C. The figure shows the slope of the regression line of the nonblind experiments and that of the combined results. The regression lines of the two kinds of measurements does not differ from each other ($t = 0.186$). Dotted lines indicate the ± S.E.M. in the slope of the regression line.

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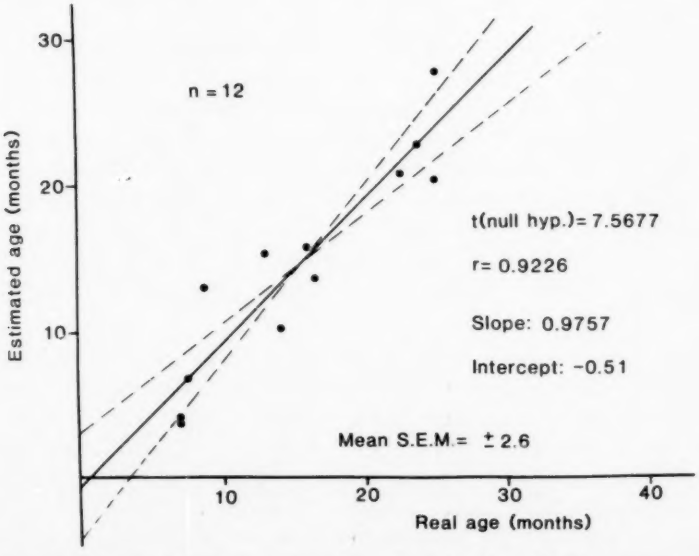

FIG. 3. Correlation analysis between the estimated age based on FRAP measurements (ordinate) and the actual one (abscissa) in male Fischer 344 rats. The linear regression line fitted to the results has a slope very near to unity (0.98). The slope differs from zero very significantly ($t = 7.57$) as revealed by variance analysis. The intercept with the ordinate differs from zero only by −0.5 months. Dotted lines indicate the ± S.E.M. in the slope of the regression line.

S.E.M. of the intercept point was ±2.6 months. This error may be considered large for young rats, however, it becomes much smaller in the older ones, when expressed as a percent of true age. Individual variations in the actual physiological state would obviously contribute to the statistical scatter and thus to the accuracy of age-estimations. Actually, to the best of our knowledge, there exists no better method for estimating age during the whole life span of rats from so few cells (28 hepatocytes/animal).

Although the method used for these experiments proved to be highly reliable, we obtained results in 4 rats which did not fit the overall pattern. These cases are described below as they seem to provide some useful observations.

1. The liver of an old (24 months) male rat showed apparent signs of fatty degeneration which was verified later by light- and electron microscopic analysis. The diffusion constant of the liver cell membrane proteins in this case was considerably higher than its age mates and higher than that of any healthy animal ($D = 3.8 \times 10^{-10}$ cm^{2}s^{-1}). At the same time, the data displayed an extremely wide statistical scatter.
2. A slightly fatty liver was noted in an adult rat (14 months) and verified by pathohistology. Although in this animal we obtained an average value of D quite near to that expected from the regression line of the male rats, the statistical scatter was so high that no correction was possible ($\chi^2 = 374.2$). Since the distribution of these data was so different from that of the normal animals, we did not include this case in our definitive age-estimation experiments.
3. In the case of a 21-month-old rat no apparent signs of fatty degeneration were seen in the liver, nevertheless, pathohistology revealed the presence of some chronic degenerative phenomena such as cholangiolar proliferation, fibrosis, vacuolization of hepatocytes, etc. In this case a value of $D = 2.58 \times 10^{-10}$ cm^{2}s^{-1} was found, corresponding to a much younger age.
4. In a 29-month-old rat with a healthy liver we measured a higher diffusion constant ($D = 2.6 \times 10^{-10}$ cm^{2}s^{-1}) than expected. The reason for this discrepancy is not resolved completely, but it is possible that this rat was improperly bled. Therefore, the liver may have contained an unusually large proportion of blood when preparing the smear. Further experiments performed with various amounts of blood added to the smears, suggest that a large D can be produced when the smear is prepared from tissues containing too much blood. This is probably the result of catalases present in the excessive amount of blood which quickly decompose the H_2O_2. Alternatively, it is possible that the unusually high D value measured in this animal is the actual value. This possibility will be discussed later.

We conclude from these four cases that liver pathologies as well as the experimental conditions (like excessive presence of blood in the tissue) may seriously influence the value of D measured by PIAF. These results indicate that D may be quite sensitive to functional alterations occurring even under physiological conditions. It should also be stressed that one has to be extremely careful in judging the role of the frequent liver alterations found in old rats (Coleman *et al.*, 1977; Goodman *et al.*, 1979; Yu *et al.*, 1982). On the other hand, the fact that such atypical results were found in 4 rats of 16, 3 of which were old, suggests that the method is reliable enough in the healthy animals and warrants further studies for eventual pathological correlations, too.

DISCUSSION

It should be emphasized first of all that the method used by us for the measurement of the lateral diffusion constant of membrane proteins (Zs.-Nagy *et al.*, 1984; 1986a) has two new characteristics, namely the method of specimen preparation and the use of PIAF. Therefore, one cannot directly compare these results with others. However, some data demonstrate that in spite of the methodological differences in our FRAP experiments as compared to those of others, one can detect considerable similarities. In particular, it seems to be important that the values of D measured by our method in the hepatocyte membrane fall in the same range as those found by others ($2–4 \times 10^{10}$ cm^2s^{-1}) using external fluorescent labels on isolated cells (De Laat *et al.*, 1980; Jacobson *et al.*, 1984; Su *et al.*, 1984).

Although there is some controversy regarding the factors determining the lateral mobility of proteins in the cell membrane (Geiger, 1983; Kleinfeld *et al.*, 1981; Tank *et al.*, 1982; Sheetz *et al.*, 1980), it seems that D is directly related to the permeability of the cell membrane. The functional significance of D has recently been analyzed in a relatively simple permeability model (Zs.-Nagy, 1986a). It was assumed that the size of the portion of the membrane impermeable for K^+ is inversely proportional to D, and that the passive potassium permeability of the membrane is a function of the size of this nonpenetrable portion. Calculations based on this assumption demonstrated that the decrease in hepatocyte membrane D observed during the life span of rats (Zs.-Nagy *et al.*, 1986a), may well explain the decrease of the passive potassium permeability measured in postmitotic cells during the aging process (see for reference: Zs.-Nagy, 1986a). This obviously supports the theory that the lateral diffusion constant of membrane proteins is a physiologically meaningful parameter which may be directly related to the permeability of the cell membrane.

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Although the physiological significance of D is yet to be established, evidence is available suggesting that D has a direct physiological role in hepatocyte functions. It has been demonstrated previously that the biliary excretion of intravenously injected ouabain decreases in an age-dependent manner in Wistar male rats (Kitani *et al.*, 1978). These results have recently been reconfirmed for F-344 rats of both sexes, the strain that has been used in both the present and previous (Zs.-Nagy *et al.*, 1986a) FRAP studies. Although the mechanism for ouabain excretion by the rat liver is not completely clear, it is most likely that some active transport mechanisms exist for both the hepatic uptake and biliary excretion processes. In this regard we have also recently observed that the velocity of ouabain uptake decreases considerably with age in isolated hepatocytes (unpublished observations). Uptake velocity is a function of receptor number and the diffusion coefficient of the carrier–substrate complex (or transfer rate constant of the carrier-ligand complex). Thus it is reasonable to assume that D directly regulates the latter parameter and eventually the uptake velocity, if PIAF includes the autofluorescence coming from the ouabain receptor protein.

Although it has not been proven directly, the hypothesis that the D is causally related to ouabain excretion is in agreement with the following experimental findings.

A 4-day spironolactone treatment of Wistar rats resulted in a highly significant increase of the hepatobiliary transport of ouabain (Kitani *et al.*, 1978, 1982), in parallel with the increase in D of the hepatocytes in 3 different age groups by about 50% (Kitani *et al.*, 1986). It is also interesting that chronic treatment of old rats with centrophenoxine caused a significant increase (about 30%) of D in the hepatocyte plasma membrane (Zs.-Nagy *et al.*, 1986b).

Some comments on the unusually high *D* value observed in a 29-month old rat may also be relevant from a functional point of view. As mentioned before in this paper, it is possible that this high *D* value is correct. There are numerous examples in the literature of very old rats with unusually efficient physiological functions of the liver. For example, a rapid increase in protein synthesis rate after 24 months of age has been reported from two different laboratories (Coniglio *et al.*, 1979; Van Bezooijen *et al.*, 1977). We have observed higher biliary transport maximum (Tm) values for sulfobromophtalein in 30-month-old rats as compared to 24-month-old values. Furthermore, we also found some 30-month-old rats which had higher biliary recovery values for ouabain than the 24-month-old ones. The apparent higher hepatic function in very old rats may be due to survival selection or to some mechanisms compensating for the urinary protein loss in old age as discussed previously (Kitani *et al.*, 1981). Although the real reason is not yet clear, unusually high *D* values observed in one 29-month-old rat (and possibly in a 21-month-old one) may correspond to physiological hyperfunctions of very old rat livers. These problems should be elucidated in future studies.

On the basis of present knowledge the results obtained can be interpreted in terms of the membrane hypothesis of aging (Zs.-Nagy, 1978, 1979, 1985; Zs.-Nagy and Semsei, 1984) as follows. The physiochemical properties of the cell membrane (i.e. the diffusibility of the components) determine basic membrane functions such as the passive permeability for potassium (and water). This parameter undergoes an age-dependent decrease from the very beginning of life due to a continuous free-radical induced and other (Zs.-Nagy, 1979) damage to membrane components. This decrease of permeability, however, causes a gradual increase in intracellular density through the increase of the intracellular potassium concentration (Zs.-Nagy, 1978). The increasing density of the cytoplasm should decrease all enzyme activities, including those involved in RNA and protein synthesis, as shown by a theoretical model of enzyme kinetics (Damjanovich and Somogyi, 1973; Somogyi and Damjanovich, 1975, 1986; Somogyi *et al.*, 1978). The cells would then reach a state in which the rate of elimination and replacement of the damaged components in the intracellular mass is considerably decreased, i.e. the protein turnover would slow down markedly (see for reference: Zs.-Nagy, 1985, 1986b). Therefore, the ratio between density and the component damage rate should deteriorate. This interpretation can be seen to be in full agreement with the requirements of theoretical gerontology (Zs.-Nagy, 1986b).

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